

**Characterisation of Nitric Oxide Synthase Activity
in the Sea Anemone *Aiptasia pallida*
and an Evaluation of Responses to Environmental
Stress**

by

Clare Emma Morrall

A thesis submitted to the University of Plymouth in
partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Plymouth Environmental Research Centre/University of Plymouth
In collaboration with the
Bermuda Biological Station for Research

May 2003

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Date.....*May 4th 2003*.....

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Studies were undertaken to investigate the presence of nitric oxide synthase (NOS; E.C. 1.14.13.39) activity in number of cnidarian species from shallow marine subtropical environments. Enzyme activity was assayed by measuring the conversion of ^3H L-arginine to ^3H L-citrulline (the citrulline assay). This assay was shown to be sufficiently sensitive for studies of this nature but was not optimised for use with cnidarian preparations. As a result, confidence in the accuracy of the generated assay results is limited. NOS activity was nevertheless demonstrated in the sea anemone *A. pallida*, in the zooxanthellae associated with *A. pallida*, *Symbiodinium* spp. and in five scleractinian coral species.

Preliminary studies were conducted to characterise NOS from *A. pallida*. NOS activity of this species was shown to be predominantly cytosolic and basal rates of enzyme activity were determined to be $0.47 \pm 0 - 9.96 \pm 0.06$ pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$. The biochemical signature of the enzyme is defined by an apparent K_m of $132.9 \mu\text{M}$ L-arginine and an apparent V_{max} of 17.7 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$. The NOS enzyme from *A. pallida* was inhibited by the arginine analogue L-NMA with an apparent K_i of $1014 \mu\text{M}$. Histochemical localisation of NOS activity by NADPH-diaphorase staining showed the enzyme to be present in the epidermal cells and at the extremities of the mesoglea.

- The hypothesis that NOS has potential as an innovative biochemical effect biomarker in cnidarian species was investigated with a number of experiments. NOS activity levels were determined in *A. pallida* previously dosed with sublethal concentrations of copper, fluoranthene or tributyltin-oxide. No clear evidence to support this hypothesis was provided by these studies. Morphological responses of *A. pallida* were monitored during exposure to contaminants, arginine analogues, a nitric oxide donor and a thermal regime that induced bleaching. Contraction of tentacles and body columns was a noted response to each of these exposures; individuals exposed to the most extreme concentrations of chemicals or the highest temperature tested typically elicited the most extreme contraction responses.

Exposure of *A. pallida* to temperatures gradually increasing from 25 to 35°C over 10 days induced bleaching characterised by reduced zooxanthellae densities. NOS activity levels and antioxidant capacity, both expressed in terms of per unit protein, were increased in anemones exposed to temperatures of $>31^\circ\text{C}$. NOS and FRAP activities expressed on a per anemone basis showed no clear change over the experimental period despite the substantial declines in host protein noted in anemones over the experimental period. These results indicate that FRAP and NOS activity levels were maintained over the course of the applied experimental thermal regime despite the occurrence of dramatic physiological changes.

Taken together, these results suggest that NOS plays an important role in the biology of cnidarians, and that as such, the activity of this enzyme has the potential to be developed into a valuable biomarker for the evaluation of initial responses of key organisms in tropical and subtropical marine ecosystems to adverse conditions. However, these results also reveal that a more comprehensive knowledge of both the roles played by NO and the responsiveness of NOS to a range of potentially adverse stimuli is required to fulfil this potential.

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Abbreviations

μE	micro einstein = $\mu\text{mol s}^{-1} \text{m}^{-1}$ per μA
Chymostatin	(N-[N α -Cabonyl-Cpd-X-Phe-al]-Phe)
DCMU	3-(3,4-dichlorophnyl)-1-1-dimethyurea
D-NMA	N ^o -Methyl-D-arginine-acetate
dpm	disintegration per minute
G	centrifugal force
Glutaraldehyde	Pentane-1, 5-dial
HB	homogenisation buffer
Leupeptin	Acetyl-Leu-Leu-Arg-al
L-NAME	N ^o -Nitro-L-arginine-methyl ester. HCl
L-NMA	N ^o -Methyl-L-arginine-acetate
L-NNA	N ^o -Nitro-L-arginine
NADPH	nicotinamide adenine dinucleotide phosphate
NR	neutral red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride)
Pepstatin A	Isovaleryl-Val-Val-statine-Ala-statine
Ppt	parts per thousand
Psi	pounds per square inch
SCUBA	self contained underwater breathing apparatus
SNAP	S-Nitroso-N-acetyl-D, L-penicillamine
SNOG	S-Nitrosoglutathione
SNP	sodium nitroprusside (sodium pentacyano-nitrosyl ferrate (II))
SS	stop solution

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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During my doctoral research I was awarded a scholarship that enabled me to attend a summer course on Coastal Pathology at BBSR in 1999.

Conference attendance and presentations

I attended the 9th Pollutant Responses in Marine Organisms (PRIMO) conference in Bergen (Norway), April 1997 where I presented a poster entitled: 'Development of nitric oxide and nitric oxide synthase as ecotoxicological biomarkers in the tropical marine environment'.

In November 1999 I attended the inaugural workshop on Indicators of Ocean and Human Health at BBSR.

My work was presented by my advisor and co-author, Dr. H. Trapido-Rosenthal, at the meeting of the Society for Integrative and Comparative Biology, in Atlanta, Georgia (USA) in January 2000. The title of the presentation was 'Nitric oxide and cnidarian-dinoflagellate symbioses: pieces of a puzzle'.

Author's Declaration (continued)

Publications

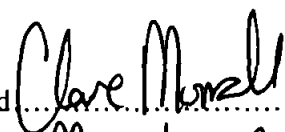
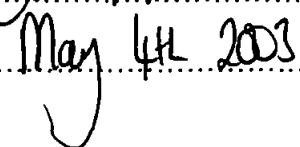
Morrall, C.E., Trapido-Rosenthal, H.G., Knap, A.H. and Depledge, M.H. Development of nitric oxide and nitric oxide synthase as ecotoxicological biomarkers in the tropical marine environment. *Marine Environmental Research*. 46, (1-5): 429-432, 1998.

Morrall, C.E., Galloway, T.S., Trapido-Rosenthal, H.G. and Depledge, M.H. Characterisation of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Journal of Comparative Biochemistry and Physiology*. 125: 483-491, 2000.

Trapido-Rosenthal, H.G., Sharp, K.H, Morrall C.E. and Galloway, T.S. Nitric oxide and cnidarian-dinoflagellate symbioses: pieces of a puzzle. *American Zoologist*. 41: 111-121, 2001.

An abstract titled 'Nitric oxide synthase activity and antioxidant status in the cnidarian *Aiptasia pallida* during artificially induced thermal bleaching' (authors: Morrall, C.E. Trapido-Rosenthal, H.G. Galloway, T.S. and Depledge, M.H.) was accepted by the organising committee of the 9th International Coral Reef Symposium. Dr Hank Trapido-Rosenthal gave an oral presentation of this paper at the conference in Bali, Indonesia in October 2000.

A manuscript titled 'Physiological responses and antioxidant status during heat stress in the tropical sea anemone *Aiptasia pallida*' is currently in preparation

Signed.....
Date.....

CHAPTER ONE

INTRODUCTION

Chapter One

Introduction

1.1 Research aims and rationale

Tropical and subtropical marine environments are at risk from a variety of natural and anthropogenic disturbances. Cnidarian-zooxanthellae associations dominate the shallow marine environments of these areas. Recent widespread coral bleaching events that are indicative of disruption of these intimate symbioses have highlighted the importance and vulnerability of these symbioses.

The aim of this project was to investigate the use of nitric oxide synthase (NOS), the enzyme responsible for generation of nitric oxide (NO), as a potential innovative biochemical biomarker for use with cnidarian-zooxanthallae symbiotic associations. Evidence supporting the concept of molecular signalling between symbionts and hosts is rapidly accumulating (Trench, 1993). NO has been identified as an important signalling molecule in a wide variety of species (for review see Lane and Gross, 1999). As such, NO is a potential mediator of symbiotic communication and it is possible that NO may be involved in the breakdown of symbiosis that results in cnidarian bleaching.

Research interest in NOS and cnidarian/zooxanthellae symbioses arose from the following observations: arginine is the precursor for NO synthesis (Palmer *et al.*, 1988); arginine dominates the free amino acid pool of symbiotic algae, whilst it is present at only low concentrations in the free-living algae (Ferrier, 1992); NO has been documented to play a role in the feeding behaviour of the cnidarian *Hydra vulgaris* (Colasanti *et al.*, 1997) and in the discharge of acontial nematocysts in the sea anemone *Aiptasia diaphana* (Salleo *et al.*, 1996). Finally, a previous report has tentatively identified NOS activity in *Aiptasia* sp. using histochemical staining (Elofsson *et al.*, 1993).

Initial experiments investigated the presence of NOS in *A. pallida* and the associated symbiont, *Symbiodinium* spp. After initial identification, a variety of biochemical parameters of the cnidarian enzyme were characterised. Histochemical staining was used to localise the enzyme activity within the animal tissue. The responses of cnidarian NOS to whole organism exposure to contaminants was investigated. Recent speculation of involvement of oxidative stress in cnidarian bleaching led to investigation of both the antioxidant capacity and NOS activity during an artificially induced bleaching event. The use of complementary cellular and whole organism biomarkers (the neutral red assay and visual response monitoring), were investigated as potential monitoring tools for use with cnidarian species.

The enzyme nitric oxide synthase was the principal biochemical parameter evaluated for biomarker potential in the research contained in this thesis. The following section introduces the nitric oxide molecule and nitric oxide synthase, the enzyme responsible for the generation of NO.

1.2 Nitric oxide and nitric oxide synthase

Nitric oxide is a diffusible, free radical gas with the chemical formula NO. Until recently nitric oxide (NO) was known predominantly as a toxic atmospheric contaminant. The biological significance of NO was initially recognized in 1986 with research that identified NO as the endothelial derived relaxing factor (EDRF) (subsequently published: Ignarro *et al.*, 1987). Over the past 15 years NO has been found to carry out more important functions than virtually any other known messenger molecule (Moncada *et al.*, 1991; Dawson and Snyder, 1994).

The radical nature of NO owes to the presence of an unpaired electron. The chemical reactivity of an atom or molecule is altered by the presence of unpaired electrons, making

them more reactive than the corresponding non-radical (Halliwell, 1994a). In biological tissues NO has a half-life of 10-60 seconds (Knowles and Moncada, 1992). NO can be interconverted among different redox forms such as the nitrosonium cation (NO^+), the nitric oxide free radical (NO^\bullet) and the nitroxyl anion (NO^-) (Beck *et al.*, 1999); each redox form has a distinctive chemistry (Stamler, 1994). At low to moderate concentrations, NO stimulates guanylyl cyclase activity and triggers the formation of cyclic GMP (cGMP), an important intracellular messenger mediating a number of physiological functions of NO such as vascular homeostasis (Beck *et al.*, 1999). At higher concentrations, NO interacts with thiol groups or transition-metal-containing proteins and can alter protein function or initiate gene expression to protect cells (Beck *et al.*, 1999; Garthwaite, 1991). These signals operate in part through redox-sensitive regulation of transcription factors and gene expression, and alter on a more long-term basis the capacity of a cell to deal with stress conditions (Beck *et al.*, 1999). NO may be simultaneously involved in signalling, cytotoxicity and cytoprotection by interaction with a variety of molecules present in the intracellular and intercellular environments (Bargmann, 1998). The net effects of NO on any given cell are either beneficial or deleterious depending on the: NO concentration obtained, duration of production, and composition of the surrounding microenvironment (e.g. availability of alternate targets and intracellular redox potential) (Bargmann, 1998).

The nitric oxide synthases (NOSs (Enzyme Commission designation 1.14.13.39)) are a group of isoforms responsible for catalysing reactions involving L-arginine, oxygen and reduced β -nicotinamide adenine dinucleotide phosphate (NADPH) that give rise to the generation of redox species of NO (NOS enzymes are synthases rather than synthetases as they do not utilise ATP). Primarily three NOS isoforms have been described: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). The nomenclature for these isoforms is based on their tissue localisation and/or responsiveness to stimuli; multiple forms may be expressed by a given cell type. Whilst other isoforms have since been described, their

general characteristics can be considered in terms of the three initially recognised types (Synder, 1994). The isoforms differ in their mode of expression and calcium dependency (Moncada and Higgs, 1993) and are the products of distinct genes (Bargmann, 1998).

The iNOS isoform is inducible while nNOS and eNOS are constitutively expressed (collectively known as cNOSs). The constitutive isoforms, nNOS and eNOS, are transiently activated by agonist-induced elevations in intracellular calcium, resulting in binding of Ca^{2+} /calmodulin (Bargmann, 1998). Inducible NOS can be induced by immunostimulants in most cell types (Bargmann, 1998). The activity of iNOS is Ca^{2+} -independent (Bargmann, 1998), requires a delay of 6-8 hours before the onset of NO production but, once induced, this enzyme is active for hours to days and produces NO in 1000-fold larger quantities than the constitutive enzymes (Beck *et al.*, 1999). NOS isoforms differ in size from 130–160 kD (Crane *et al.*, 1997) but they all share the same general three-component structure. They each have an NH_2 -terminal catalytic oxygenase domain that binds heme (iron protoporphyrin IX), tetrahydrobiopterin and the substrate L-arginine; a COOH-terminal reductase domain that binds the cofactors flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and NADPH and a calmodulin-binding region that regulates the flow of electrons between the oxygenase and reductase domains (Stuehr and Marletta, 1997). NOS isoforms are homodimers and the catalytic mechanism of each isoform appears to be the same (Crane *et al.*, 1997). Recent phylogenetic evidence indicates that whilst the structure of the enzyme has in general been highly conserved among isoforms, the gene duplication leading to the inducible form appears to have occurred after the divergence of vertebrates and arthropods (Hughes, 1998).

Redox species of NO (NO^\bullet , NO^- and NO^+) are generated by NOS isoforms in a two-step heme-based oxygenation of L-arginine (figure 1.1). In the first step, a mixed function oxidation-type reaction leads to the formation N-hydroxy-L-arginine (NOH-Arg) (Sono *et al.*, 1996). This stable intermediate is further oxidised via the formation of a dioxygen iron

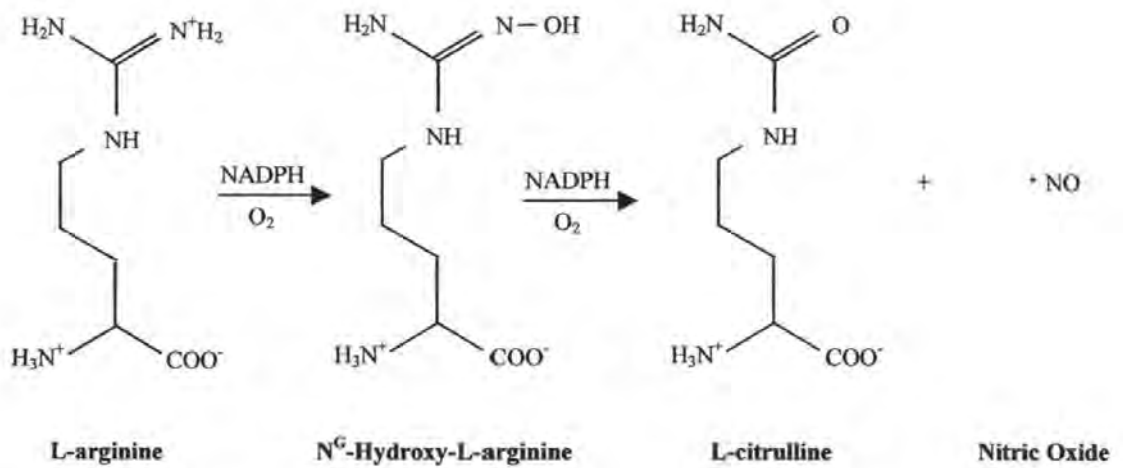


Figure 1.1. The two step conversion of L-arginine to L-citrulline and NO catalysed by NOS. Step one converts L-arginine to N^G hydroxy-l-arginine, which is subsequently oxidised to form citrulline and NO in step two.

species incorporating one electron each from NADPH and NOH-Arg and an O₂ molecule. The resulting NOH-Arg radical is attacked at its guanidino-carbon, leading to the incorporation of one oxygen and the cleavage of C-N to yield NO (Nathan and Xie, 1994). Synthesis of NO is accompanied by the stoichiometric production of L-citrulline. Traditionally, despite little direct evidence, NO[•] was considered to be the proximal product of the NOS catalysed reaction. Controversy currently exists in this area of research, NO[•] has been both ruled out (Schmidt *et al.*, 1996), and confirmed as the direct product of NOS (Xia and Zweier, 1997).

The combination of roles played by NO, i.e. intracellular signal, transcellular signal and cytotoxic molecule, is unprecedented in biology (Knowles and Moncada, 1994). The sensitivity to NO varies from one cell to another (Hibbs *et al.*, 1990) and it can have opposing effects depending on the concentration and the sites from which it is released (Kolb and Kolb-Bachofen, 1992).

The presence of NO is well demonstrated in vertebrates (Colasanti and Venturini, 1998) and a myriad of roles for NO and redox forms of NO have been identified. These include the fundamental processes of blood pressure regulation (Jia *et al.*, 1996), neurotransmission (Synder, 1994; Ignarro *et al.*, 1987) and immune response (Moncada *et al.*, 1991). In contrast to the role in normal physiological functions, NO is also recognised to play a part in the pathogenesis of conditions as diverse as septic shock, stroke and neurodegeneration (Colasanti and Venturini, 1998).

The first clear evidence of NO production in invertebrates was provided in 1991 by Radomski *et al.*, who identified NOS in the haemocytes from the arthropod *Limulus polyphemus*. Nitric oxide has since been identified in a wide variety of organisms including plants (Delledonne *et al.*, 1998) rats (Giovanelli *et al.*, 1991), locusts (Elphick *et al.*, 1993) and sea anemones (Salleo *et al.*, 1996). The roles of nitric oxide in invertebrates are closely related to the physiological actions described in vertebrates (Martinez, 1995) e.g.

neurotransmission, neuromodulation, smooth muscle activity and immunological defence mechanisms (Colasanti and Venturini, 1998). Experimental evidence suggests the presence in invertebrates of new NOS isoforms different from those known for higher organisms (Colasanti and Venturini, 1998).

The organisms used in this research were tropical/subtropical species; the following section outlines the physical and biological characteristics of these environments. An overview of the importance of, and threats posed to these environments is included.

1.3 The tropical and subtropical marine environment

‘the tropics are complex and our understanding of these systems are woefully imperfect’

Michael A. Mares, 1997.

The world’s tropics lie along the equator, extending north and south to 23° of latitude. They are adjoined in both hemispheres by the subtropics, which are defined as much by their characteristic temperatures as they are by degree of latitude. The tropics and subtropics have relatively high sea temperatures with oceanic surface water typically between 26°C and 30°C. The tropical ocean includes almost 50% of the world’s open water and 30% of the total area of continental shelf (Longhurst and Pauly, 1987). As such, the tropical marine environment is a major portion of the world’s ocean.

The extent of marine biogeographical regions can be related more closely to the course of isotherms than to any other factor (Belda-Baille *et al.*, 1998). Water temperature exerts a major control over the distribution and activities of marine organisms. Temperature tolerances differ widely between species, but each is restricted in distribution within its particular temperature range. Greater thermal tolerance ranges are prevalent in temperate regions, while smaller ranges are more prevalent in polar and tropical waters (Moore, 1972); as a direct result of this, temperatures outside those normally encountered are thought to be of greater biological significance in the tropics than in temperate zones.

The physical and biological characteristics of tropical marine environments differ markedly from those of their temperate counterparts. Some of these notable differences are detailed in table 1.1.

Temperature	higher mean
	lower annual thermal range
	thermal maximum closer to ambient temperature
Light	higher total received annually
	lower annual range of input
	lower annual range in day length
Dissolved oxygen	lower
Water clarity	higher
Metabolic rates	higher at ambient temperatures
Growth rates	higher
Thermal tolerance	smaller range

Table 1.1. Generalised physical and biological differences of shallow tropical marine ecosystems compared to their temperate counterparts at comparable depths (modified from Hatcher *et al.*, 1989).

As much as 75% of global biodiversity is contained in tropical ecosystems (Lacher and Goldstein, 1997), yet our knowledge of these ecosystems lags significantly behind that of the temperate environment. The islands and coastal waters of the tropics and subtropics possess unique, species rich, and highly productive ecosystems, including mangrove forests, seagrass meadows, and coral reefs (Peters *et al.*, 1997). Coral reefs are among the most biologically diverse ecosystems on our planet and are noted for some of the highest levels of total gross productivity on earth (Bryant *et al.*, 1998).

The environments of the tropics and subtropics were long considered pristine and safe from the degradation of human activities. Research conducted during the last 20 years has shown

however that chemical contaminants are present in the water, sediment, and biota of tropical marine ecosystems (Peters *et al.*, 1997). Tropical ecosystems are under increasing threat of development and habitat degradation from population growth and urbanisation, agricultural expansion, deforestation and mining (Lacher and Goldstein, 1997). To date, ecotoxicology has focused almost exclusively on countries and ecosystems in temperate zones (Lacher and Goldstein, 1997); tropical and subtropical ecosystems have been largely neglected. Although tropical and subtropical environments differ from their temperate counterparts in their physical, chemical and biological attributes (Lacher and Goldstein, 1997), their constituent flora and fauna appear to be at least as sensitive to the effects of toxic chemicals as are temperate and cold water species (Peters *et al.*, 1997). The fate, pathways and effects of chemicals in these environments have barely begun to be addressed (Depledge, 1984). At higher temperatures, solid and liquid pollutants are more soluble and metabolic rates are faster, therefore rates of biological uptake of pollutants are likely to be higher in warm tropical waters. However, rates of excretion and degradation (biological and physicochemical) are also likely to be faster at higher temperatures. Despite the fundamental differences that exist between climatic regions, ecotoxicological and risk assessment techniques and procedures developed for temperate environments are often applied to environments beyond the climatic range for which they were intended. At present there are insufficient comparative data on the metabolism and toxicity of most pollutants to draw useful conclusions about their consequences in tropical versus temperate marine ecosystems (Hatcher *et al.*, 1989).

The organisms used in this research were obtained from the marine environment of Bermuda. For this reason, an overview of the geography, environmental characteristics, and marine life of Bermuda is presented here.

1.4 The environment of Bermuda

'You go to heaven if you want to - I'd rather stay here in Bermuda'

(Mark Twain, 1910).

The country of Bermuda consists of a group of oceanic islands situated in the western north Atlantic at 32° 20 N and 65° 45 W. The archipelago is approximately 960 km southeast of Cape Hatteras on the east coast of the USA, 1500 km north of the Caribbean. Geologically, Bermuda is a volcanic seamount that rises 4 km from the ocean floor. A fossilised limestone cap known as the Bermuda Platform covers 500 km² (figure 1.2). The 120 islands that make up Bermuda lie on the southeast of the platform and cover an area of 55 km². Extensive reef growth occurs off the northern shore of the islands, a narrow strip of reef runs along the southern shore close to the platform edge.

The climate and seawater temperatures experienced on and around Bermuda are typical for an island located in a subtropical gyre. Air temperatures range from 10.5-32.5°C; oceanic seawater temperatures range from 19.0-30.0°C while inshore waters experience a wider temperature range of 14.0-35.0°C (pers comm. Bermuda Weather Service). The tidal range averages 75 cm (mean water level difference between high and low tide) and salinity is approximately 36 parts per thousand.

The marine biota of Bermuda is largely subtropical and Caribbean in origin (Cavaliere *et al.*, 1992). The shallow waters of the Bermuda platform contain extensive coral reef formations; seagrass meadows and mangrove forests are found at a number of locations around the islands coasts. Bermuda has the most northerly coral reef system in the Atlantic. Species diversity is reduced relative to the Caribbean owing to the exclusionary effect of the low winter water temperatures and the selective nature of the 1500 km oceanic journey that the larvae of potential immigrants must withstand in order to recruit successfully in Bermudian waters. Reef formation is conspicuous however and the reefs are composed of, and populated by, an abundant coral fauna (Dodge *et al.*, 1982).

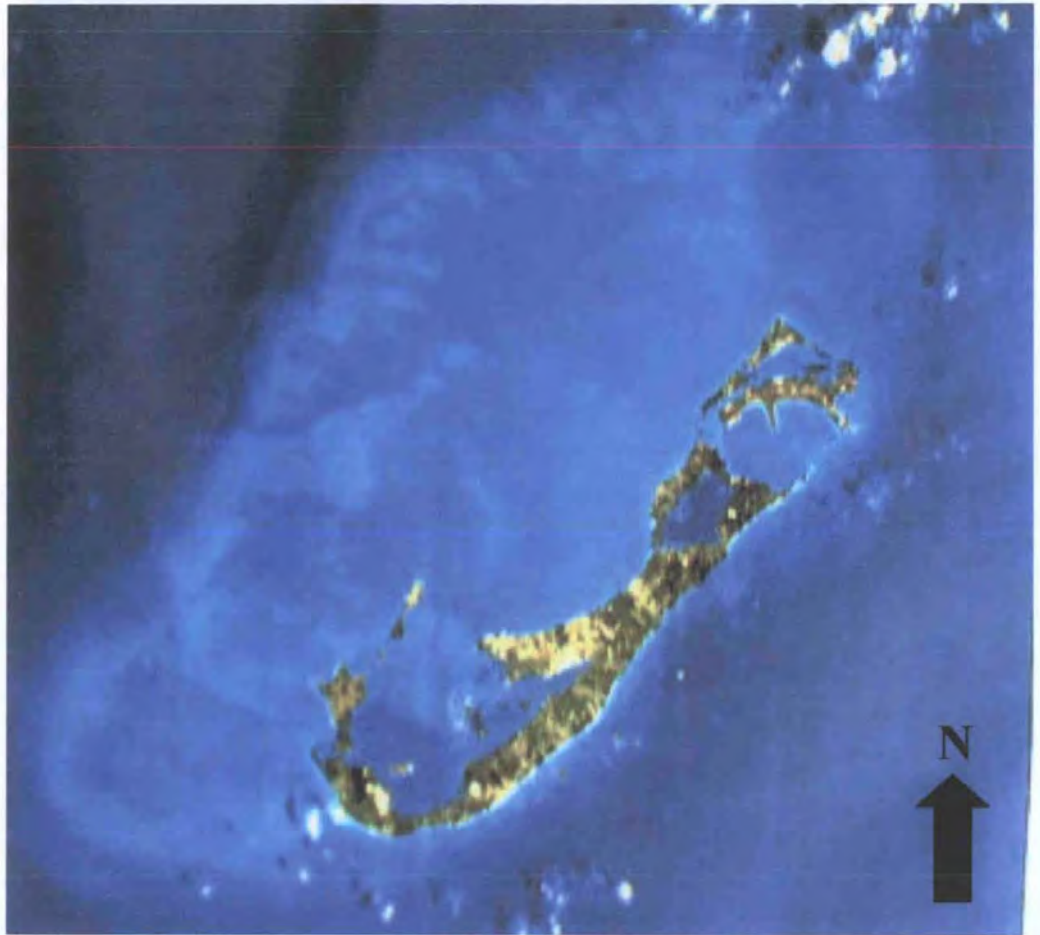


Figure 1.2. The Bermuda Platform from 320 miles. Exposed land is shown in green/yellow. Shallow waters in which scleractinian and alcyonarian corals proliferate are shown in light blue. Deep oceanic water, indicated by dark blue, surrounds the Bermuda platform. Reproduced with permission from the Bermuda Aquarium, Museum and Zoo.

Natural disturbances experienced by Bermuda's environment are meteorological in nature, and are dominated by the effects of hurricanes. Winds, waves and precipitation associated with these weather systems affect both terrestrial and marine habitats. Bermuda supports a dense human population, with approximately 60, 000 inhabitants. The country is a highly developed nation with a high income per capita. The environment is subject to anthropogenic influences on the environment resulting from the population's transportation requirements, domestic waste disposal and pesticide and fertilizer runoff from the islands agricultural land and numerous golf courses. In addition, a shoreline metals dump, and dredging activities associated with land reclamation and shipping channel maintenance both contribute to anthropogenic environmental disturbance.

One of the aims of the research contained in this thesis was to provide information that would improve ecological risk assessment in the tropical and subtropical marine environments. A biomarker-based approach was used. The following section introduces the principals of ecotoxicology, ecological risk assessment and the biomarker approach.

1.5 Ecological risk assessment and the biomarker approach

'Because humans are evolutionarily programmed to respond to sudden dangers, we have difficulty perceiving small, gradual changes in our surroundings'

Ornstein and Ehrlich, 1989.

Ecotoxicology is the study of the interactions between contaminants and biota, including toxic effects and interactions, with processes controlling ecosystem function (Fent, 1996). Over nine million chemicals are listed on the Chemical Abstract Service's Registry of Chemicals, and an estimated 75-80, 000 are in daily use (Fent, 1996). These chemicals are potential contaminants and pollutants of the global ecosystem (Depledge and Fossi, 1994), and pose risks to both ecosystems and human populations. Few chemicals in daily use have been assessed for potential effects on ecosystems (Fent, 1996).

Ecological risk assessment is the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems are estimated with a known degree of certainty using scientific methodologies (Depledge and Fossi, 1994). In the past, risk assessment has largely consisted of analysis of abiotic environmental parameters such as water quality, sediment chemistry and toxicity tests performed on organisms. While the tools and analytical capabilities for documenting the presence of contaminants in marine environments and in organisms are well developed, data of this sort give little or no information as to the risk or actual harm posed to the living components of the ecosystem. As environmental risk assessment should primarily highlight the dangers posed to the living components of the environment, it is important to focus on organismal responses to contaminant exposure. The study of the biological effects induced by pollutants is a fundamental approach to assessment of the impact of anthropogenic disturbances (Regoli *et al.*, 1998a). Toxicity testing monitors the responses of organisms to contaminant exposure, but often the lethality of different concentrations of chemicals is assessed using death as an end point. Lethality is a precise and unequivocal measure, but is of little use in the study of chronic effects or from the standpoint of remediation.

In recent years, direct measurements of contaminant effects on organisms have been developed as useful tools for risk assessment. These direct measurements are known collectively as biomarkers. Biomarkers are biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples or at the level of the whole organism, that provide evidence of exposure to and/or effects of one or more chemicals, pollutants and or radioactivity (NRC, 1987). Cellular and biochemical responses to environmental stress can often be detected before a more integrated toxicity becomes apparent in the physiology of the whole organism (Regoli *et al.*, 1998a). Alterations in cellular and biochemical biomarkers may be mirrored by changes seen at higher levels of organisation (e.g. physiologically, behaviourally or at the population level). Monitoring

sublethal biological effects has the potential to highlight contaminant impacts before lethality is imminent, when remediation is still feasible.

Whilst a measure of a contaminant in the water column stands alone as evidence of contamination, a biomarker has the potential to give not only an indication of exposure, but may also give information on the biological effect and impact. The underlying basis of all contaminant-induced pathological change is damage or perturbation of living processes at the molecular and subcellular levels of biological organisation (Slater, 1978). Detection of changes at these basic levels has the potential to provide markers of initial responses. Early detection will provide the maximum time frame for action to be taken to prevent or ameliorate further deleterious effects. It is unlikely that a single biomarker will be able to adequately predict either response or exposure to contaminants (either singly or in combination). For this reason, suites of biomarkers should be utilised to provide robust indicators of the biological status of organisms. Biomarkers should if possible, be related to a given degree of impairment of growth, reproductive output or energy utilisation. These are measures of an organism's fitness that directly affect the survival of the organism in question. Impairment of any fitness parameter should be attributed to exposure to a known amount of a specific pollutant.

Before using biomarkers to assess contaminant exposure and/or effects, rigorous testing and validation is required. In addition, the following considerations must be addressed. Many biomarker responses are transient; it is therefore necessary to establish in advance what duration of response can be expected. Often background knowledge of potential biomarkers is lacking, necessitating careful characterisation of the parameter under 'normal' conditions before observing pollutant-induced change. Detection of pollutant induced changes at organism and community levels require knowledge of normal patterns of change, often necessarily collected from long term monitoring studies. At the biochemical level, knowledge of normal conditions is still essential but as changes may be detected within

hours of the application of a disturbance, the need for long term monitoring data may be eliminated. As such, monitoring lower levels of organisation such as biochemical and cellular change offers the opportunity of developing rapid indicators of pollutant-induced change.

The following limitations may apply to the use of specific biomarkers: inherent biological variability may mask a pollutant response; an understanding of biological mechanisms and systems is required in order to assess the significance of the biomarker response observed: extrapolation of single-species responses to other organisms or species may be inappropriate and inter-individual variation of biochemical parameters can be confounded by age, sex, circadian rhythm, nutritional status and seasonality. As a result of these influences, careful experimental design is required.

The following section outlines the rationale for selection of *A. pallida* as research organism for the majority of the research detailed here.

1.6 Species selection rationale

Invertebrates represent 95% of all animal species in natural ecosystems and are the key structural and functional components of the marine ecosystem (Depledge and Billingham, 1999). The frequent choice of invertebrates in biomarker studies reflects their ecological importance. Furthermore, since many biochemical and physiological systems are common to all organisms, results obtained from the study of invertebrate biomarkers may be broadly applicable to other organisms in the environment under investigation.

Jellyfish, hydra, sea anemones and corals are grouped together in the phylum cnidaria. Sea anemones and their close relatives the corals are among the most abundant representatives of coastal benthic communities, especially in the tropics (Mercier *et al.*, 1996). Scleractinian corals dominate the shallow marine environments of the tropics and subtropics. These corals

provide and maintain the structural framework that provides habitats and feeding grounds for a profusion of organisms. Coral reefs are the most biologically diverse of all shallow water marine ecosystems (Roberts *et al.*, 2002). Owing to their high productivity, immense biological wealth and the economic and environmental services provided to millions of people, coral reefs are amongst the most valuable ecosystems on earth (Bryant *et al.*, 1998). The value of the living resources and services coral reefs provide has been estimated at US \$375 billion per year or US \$6075 ha⁻¹ yr⁻¹ (Costanza *et al.*, 1997). This value notwithstanding, it has been calculated that human activity potentially threatens 58% of the world's coral reefs (Bryant *et al.*, 1998). The proximity of coral reefs to the coast exposes them not only to subsistence pressures but also to other anthropogenic stresses such as pollution (in the form of industrial, chemical and sewage waste) and sedimentation (resulting from land clearing, dredging, land reclamation and mining).

Anemones and corals are characterised by a high surface area to volume ratio (resulting from their numerous tentacles), and by soft bodies sustained by a hydrostatic skeleton. These features, along with their predominantly sessile nature and opportunistic predatory and plankton feeding lifestyle, makes these animals particularly vulnerable to water borne contaminants. Owing to their ecological importance, the risks posed to them and their vulnerability, sea anemones and corals are suitable test organisms in aquatic toxicology.

The sea anemone *Aiptasia pallida* (= *A. tagetes*, sensu Verill 1907 (Sterrer, 1986)) was the organism used in the majority of this research. *A. Pallida* is closely related to scleractinian corals both taxonomically and physiologically. In addition, the species is naturally associated with the zooxanthellae *Symbiodinium* spp., the same species found in the majority of tropical and subtropical cnidarians (Trench, 1993). Both *A. pallida* and its pacific congener, *A. pulchella*, survive long periods in culture without food, can be maintained either with or without their naturally occurring algal symbionts (symbiotic and aposymbiotic forms respectively) and the species asexual mode of reproduction enables

generation of clones. These attributes have resulted in *A. pallida* and *A. pulchella* being a popular choice of experimental cnidarian. As a consequence, a wealth of literature is available relating to these species and many aspects of the species biology have been documented. A review of the biology of *A. pallida* and *A. pulchella* is provided in section 1.8.

The following section provides an overview of what is currently known about cnidarian responses to a variety of stresses. A summary of previously documented stress responses, a discussion of the deficiencies of current knowledge in this field and suggestions of the priorities to be addressed by future research are provided.

1.7 Cnidarian stress responses

A number of reviews covering aspects of stress in tropical marine species and environments have been published in the last 30 years (Moore, 1972; Stebbing and Brown, 1984; Brown and Howard, 1985, Kinsey, 1989; Peters *et al.*, 1997; Hughes and Connell, 1999). The effects of a range of contaminant and physical stresses have been observed in a variety of cnidarian species. A summary of noted cnidarian responses to chemical or physical stresses is presented in table 1.2.

It is clear from this tabulated summary that a wide range of stress responses have been observed in cnidarian species. A number of the noted stress responses, i.e. morphological changes and alterations in mucus production can be assessed visually in intact organisms; these responses have been utilised in toxicant bioassays (Blaise and Kusui, 1997; Trottier *et al.*, 1997).

Contaminant/physical disturbance	Noted effects
Increased irradiance	Zooxanthellae density reduction (Hoegh-Guldberg and Smith, 1989; McCloskey <i>et al.</i> , 1996)
	*Increased specific activities of SOD, catalase and ascorbate peroxidase (Lesser, 1989)
	*Photoinhibition of catalase (Tapley <i>et al.</i> , 1998; Tapley, 1998)
	*Reduced chlorophyll per zooxanthellae (Lesser, 1989)
Reduced irradiance	Reduced biomass (Muller-Parker, 1985)
	Zooxanthellae density increase (Saunders and Muller-Parker, 1997)
Increased UV radiation	*Inhibition of photosynthesis (Shick <i>et al.</i> , 1995; Lesser, 1998)
	Zooxanthellae loss (Hannack <i>et al.</i> , 1997)
	*Reduced zooxanthellae mitosis (Hannack <i>et al.</i> , 1997)
	*Reduced chlorophyll per zooxanthellae (Lesser, 1996; Lesser, 1989)
	Zooxanthellae ultra structural abnormalities (Hannack <i>et al.</i> , 1997) or changes (Muscantine <i>et al.</i> , 1991)
	*Increased specific activities of SOD, catalase and ascorbate peroxidase (Lesser, 1989; Lesser, 1998)
	*Production of mycosporine-like amino acids (Shick <i>et al.</i> , 1995; Banaszak and Trench, 1995)
	Zooxanthellae density reduction (Hoegh-Guldberg and Smith, 1989; McCloskey <i>et al.</i> , 1996)
	*Increased specific activities of SOD, catalase and ascorbate peroxidase (Lesser, 1989)
	*Photoinhibition of catalase (Tapley <i>et al.</i> , 1998; Tapley, 1998)
	*Reduced chlorophyll per zooxanthellae (Lesser, 1989)
Reduced salinity	Zooxanthellae loss (Engebretson and Martin, 1994)
Drilling mud	*Changes in free amino acid pools (Powell <i>et al.</i> , 1984)
	Reduced calcification rate (Powell <i>et al.</i> , 1984)
	Increased bacterial aggregates (Krone and Bigg, 1980)
	*Protein and amino acid loss (Powell <i>et al.</i> , 1984)
Increased sedimentation	Lowered skeletal density and calcification (Dodge and Brass, 1984)
	Tissue discoloration (Wesseling <i>et al.</i> , 1999)
	Morphological changes (Blaise and Kusui, 1997; Trottier <i>et al.</i> , 1997)

Elevated temperature	Reduction in zooxanthellae density (Hoegh-Guldberg and Smith, 1989; Hayes and King, 1995; Miller <i>et al.</i> , 1992; Lesser <i>et al.</i> , 1990; Lesser, 1997; Warner <i>et al.</i> , 1999)
	*Loss of zooxanthellal photosynthetic pigment (Hoegh-Guldberg and Smith, 1989).
	*Heat shock protein induction (Miller <i>et al.</i> , 1992; Black <i>et al.</i> , 1995; Branton <i>et al.</i> , 1999; Hayes and King, 1995; Sharp <i>et al.</i> , 1994; Bosch <i>et al.</i> , 1988)
	Morphological changes (Black <i>et al.</i> , 1995)
	*Reduced or absent photosynthesis (Hoegh-Guldberg and Smith, 1989; Iglesias-Prieto <i>et al.</i> , 1992; Smith <i>et al.</i> , 1988; Lesser, 1997; Warner <i>et al.</i> , 1999)
	*Decline in lipid levels (Glynn <i>et al.</i> , 1985a)
	*Increased respiratory rate in host and zooxanthellae, (Hoegh-Guldberg and Smith, 1989; Lesser, 1996)
	*Increased abundance of a novel 33 kDa protein in zooxanthellae (Bythell <i>et al.</i> , 1995)
	Zooxanthellae density unchanged (Saunders and Muller-Parker, 1997)
	*Reduced levels of UV absorbing compounds (Lesser <i>et al.</i> , 1990)
	*Production of superoxide (Nii and Muscatine, 1997)
	Reduced fertilization (Omori <i>et al.</i> , 2001)
	*Increased levels of SOD and hydrogen peroxide in zooxanthellae (Lesser, 1996; Lesser <i>et al.</i> , 1990)
	*Lipid peroxide levels, malondialdehyde, 4-hydroxy-2-trans-nonenol, total glutathione, heat shock proteins 60 and 70, α B-crystallin homologue, chloroplast small heat-shock protein, homologues to the plant class I-IV small heat-shock proteins, Mn and Cu/Zn superoxide dismutases, metallothioneins, specific P450 family members, protein carbonyl and ubiquitin significantly affected (Downs <i>et al.</i> , 2000)
Reduced temperature	Zooxanthellae loss (Muscatine <i>et al.</i> , 1991; Steen and Muscatine, 1987)
Mixed land based pollution stress (combined industrial effluent, sewage, sedimentation)	Reduced species diversity (Edinger <i>et al.</i> , 1998)
	Reduced live coral coverage (Edinger <i>et al.</i> , 1998)
	Absent growth (Edinger <i>et al.</i> , 1998)
Crude oil	Inhibition of larval metamorphosis and settlement (Kushmaro <i>et al.</i> , 1997)
Sewage	No apparent negative impact (Grigg, 1994)
Alizarin	Depression in calcification rate (Dodge <i>et al.</i> , 1984)
Pemethrin/dibrom	*Differential mRNA expression (Morgan and Snell, 2000; Morgan <i>et al.</i> , 2001)
Cyanide	Zooxanthellae loss (Jones and Hoegh-Guldberg, 1999)

	*Photosynthetic inhibition and impairment (Jones and Hoegh-Guldberg, 1999)
Heavy Metals	Zooxanthellae loss (Miller <i>et al.</i> , 1992 (Cu), Jones, 1997 (Cu), Harland and Brown, 1989 (Fe))
	*Heat shock protein induction (Miller <i>et al.</i> , 1992 (Cu))
	Skeletal accumulation of heavy metals (Guzman and Jimenez, 1992 (Al, Cr, Mn, Fe, Cu, Zn, Cd, Pb, V, Ni))
	Reduction of gamete fertilization success (Reichelt-Brushett and Harrison, 1999)
	Increased mucus production (Harland and Nganro, 1990 (Cu), Mercier <i>et al.</i> , (TBT))
	Morphological changes (Harland and Nganro, 1990 (Cu))
	*Differential mRNA display (Morgan and Snell, 2000; Morgan <i>et al.</i> , 2001 (Cu))
Herbicides	Tissue sloughing (Glynn <i>et al.</i> , 1984)
	Mucus production (Glynn <i>et al.</i> , 1984; Mercier <i>et al.</i> , 1998)
Polycyclic aromatic hydrocarbons	*Production of oxidative metabolites (Winston <i>et al.</i> , 1998 (Benzo[a] pyrene))
Butyltin	Zooxanthellae loss (Mercier <i>et al.</i> , 1996 and 1997)
	Stimulated mucus production (Mercier <i>et al.</i> , 1997 and 1998)
	Increased bacterial aggregates (Mercier <i>et al.</i> , 1997)
	Thickening of pedal disc ectoderm (Mercier <i>et al.</i> , 1997)
	Morphological changes (Mercier <i>et al.</i> , 1996 and 1997)
	Decreased numbers of undischarged nematocysts (Mercier <i>et al.</i> , 1997)
	Pedal disc detachment (Mercier <i>et al.</i> , 1997)

Table 1.2. Review of noted effects in cnidarian species exposed to a range of conditions. Noted effects generally refer to host, unless otherwise stated. Photosynthetic effects refer to zooxanthellae. Elevated and reduced temperature conditions typically relate to those at the extremes or beyond the normally encountered thermal range. Asterisks mark biochemical or molecular responses.

Stress induced changes in zooxanthellae physiology have been frequently noted and reductions of zooxanthellae densities that may ultimately result in bleaching have been observed in response to a wide range of stressors. Studies of cnidarian-symbiodinium stress responses at the biochemical or molecular level are limited to a small number of investigations conducted in the past 18 years; these studies are marked with asterisks in

table 1.2. The observed biochemical and molecular parameters include: changes in the production or activities of oxidants or antioxidants in both host and zooxanthellae, heat shock protein induction, production of specific amino acids and a number of aspects of zooxanthellae photosynthesis ranging from general parameters (such as changes in pigment levels) to highly specific parameters (i.e. observation of damage to photosystem II). Work published recently has observed contaminant responses at the mRNA level. Studies of this kind that observe changes in gene expression resulting from exposure to stressors have potential to provide highly specific, sensitive and rapid effect biomarkers which represent the initial step in a stress response. In addition biomarkers of this kind may give an insight of the mechanism underlying the stress responses.

While documentation of anthropogenic impacts in cnidarian species is undoubtedly improving, advancement of our understanding of the mechanisms of effect, and their consequences is currently inadequate. Information is lacking with regard to long-term effects, appropriate indicator species, and biomarkers for tropical communities (Peters *et al.*, 1997).

Reductions of zooxanthellae density (bleaching) is the most widely reported cnidarian response to a wide range of stressors, yet we understand little of the mechanisms that lead to the breakdown of the intimate association between host (cnidarian) and symbiont zooxanthellae. Few studies have investigated bleaching at the cellular or biochemical level and it remains to be seen if the mechanism of release is uniform. Owing to the intimate relationship between cnidaria and zooxanthellae, responses of both partners to stressors should be monitored in parallel and the possible interplay of these responses requires investigation.

There is an urgent need to improve our ability to assess the health status of cnidarian species that dominate the shallow marine environments of the tropics and subtropics: this need was addressed by the research detailed in this thesis. A range of approaches were made

including: visual assessment of intact organisms, assessment of cellular structures and monitoring biochemical parameters (i.e. NOS activity and antioxidant capacity). A component of this research targeted the elucidation of the biochemical and/or molecular mechanisms that underlie bleaching. This research was motivated by a need to improve understanding of stress responses in cnidarian/zooxanthellate symbioses and to provide biomarkers that enable assessment of the health status of cnidarian species.

An overview of the biology of *Aiptasia pallida*, the species used in the majority of the research contained in this thesis, is provided in the following section.

1.8 The biology of the sea anemone *Aiptasia pallida*

For elegance and grace, for delicacy of form and quiet beauty of colour, the sea anemones have a charm unrivalled by any other form of life; they are the flowers of the reef'.

T.C. Roughley, 1954.

Approximately 1000 species of sea anemone exist, ranging in size from a few centimetres to several meters across. They are exclusively marine and are found in all the oceans of the world, from the inter-tidal to depths of thousands of meters. Anemones are largely solitary and normally live attached to some sort of solid object. Taxonomically *Aiptasia pallida* is classified as follows: Phylum cnidaria, class Anthozoa, subclass Hexacorallia, order Actinaria and family Aiptasiidae. The species is known in Bermuda as the common mangrove anemone, the pale anemone or the white speckled anemone (Verill, 1907). Figure 1.3 illustrates an example of *A. pallida*. The stalk of *A. pallida* is typically 0.5-3 cm in diameter, with a ring of smooth tentacles that vary in length in proportion with the stalk size (range approximately 0.5-3 cm). *A. pallida* is semi-transparent; coloration is derived from photosynthetic pigment contained in symbiotic zooxanthellae (located within gastrodermal cells) and ranges from pale to dark brown. The oral disc and tentacles may be speckled with tiny cream/bluish dots, which are at times arranged in circular bands. *A. pallida* is found in



Figure 1.3. The common mangrove anemone *Aiptasia pallida*. Scale bar indicates approximately 0.5 cm.

the marine environments of Bermuda, Florida, the Bahamas and the Caribbean. The species inhabits protected bays and inlets and can be found among stones and algae, on sponges and mangrove roots (Sterrer, 1986). *A. pulchella*, the species congener of *A. pallida*, is present in tropical and subtropical Pacific waters.

Cnidarians are simple animals containing few cell types. Members of the phyla cnidaria have tissue grade construction; their cells are organised into discernible tissues having specialised functions, but the tissues are not further grouped into organs having multiple tissue types (Shick, 1991). Experimental analysis of whole anemone preparations is often appropriate as a result of the tissue grade construction. Anemones consist of three major parts, the oral disc, the column and the pedal disc (shown in figure 1.4). Structurally, anemones are elastic, muscular cylinders open at the oral end, and full of seawater, which may account for over 80% of their live weight (Shick, 1991). The body cavity is the gastrovascular cavity that has a single opening (the mouth) on the oral surface.

The epidermis of anemones is composed of a thin outer and inner layer of cells (ectoderm and endoderm respectively). Between these layers is mesoglea, a thick substance containing a collagenous fibre system similar to certain types of connective tissue found in vertebrates. The epithelium is composed of three strata: an outer wide stratum comprising the main portions of the supporting cells interspersed with gland and sensory cells, a nervous stratum adjacent to the bases of these cells, and an innermost muscle stratum next to, or embedded in, the mesoglea (Hyman, 1940).

The mouth is an elongate slit located in the centre of the peristome, a smooth area of tissue that extends to the proximal end of the tentacles. The area of the mouth and peristome is known as the oral disc. The mouth opens into a flattened, ectodermal invagination known as the pharynx, which in turn opens into the gastrovascular cavity. The flat pharynx and the linear mouth destroy the otherwise perfect radial symmetry of the anemone and result in a bi-radially symmetrical body plan (Shick, 1991).

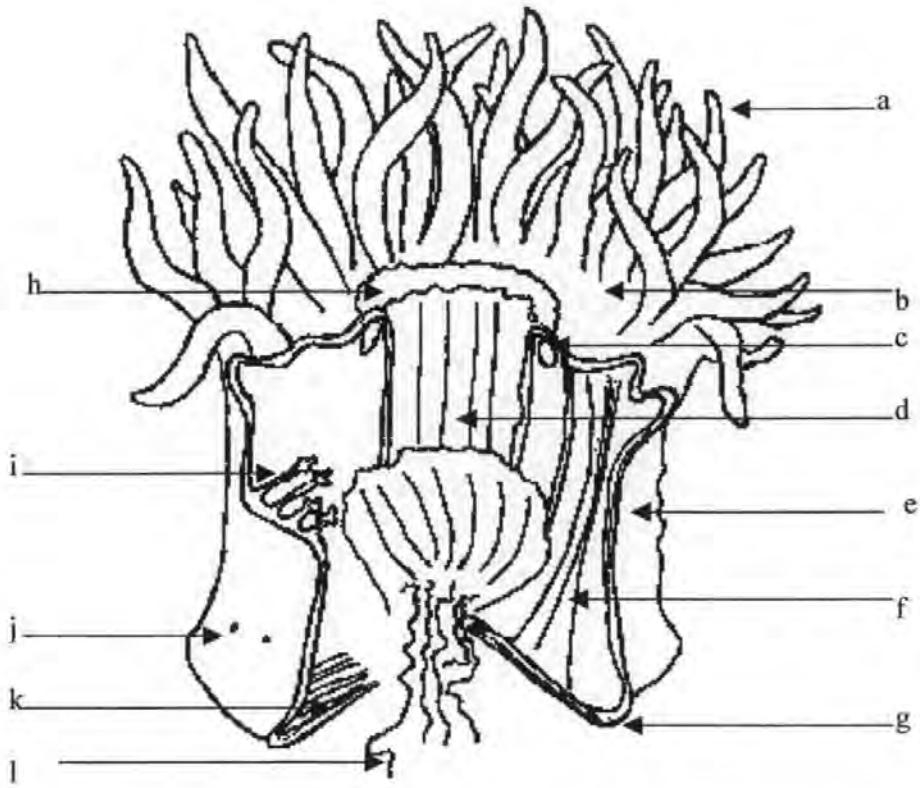


Figure 1.4. Diagrammatic representation of the anatomy of *Aiptasia pallida*. Labels: a= tentacles, b= oral disc, c= oral stoma, d= pharynx, e= epidermis, f= retractor muscle, g= pedal disc, h= mouth, i= mesenteries, j= cinclides, k= parietobasilar muscle, and l= acontia. Modified from Shick (1991).

Anthozoans possess a pharynx or stomodaeum. This distinguishes them from all other members of the cnidarian phyla. The pharynx consists of a tube lined with invaginated ectoderm that hangs down into the mouth and through to the gastrovascular cavity. The pharynx serves chiefly in the ingestion of food.

Mesenteries (known alternatively as septa) project inward from the body wall as shown in figure 1.4. They are the sites of digestion, absorption, and gamete development and are important in defensive reactions (Shick, 1991). As such, they are central to cnidarian biology. Septa are composed of gastrodermis and mesoglea and occur as mirror image pairs on opposite sides of the directive axis. They are fastened at their upper end to the oral disc and pharynx, and divide the gastrovascular cavity longitudinally into compartments or endocoelic chambers. Adjacent to each of these chambers is an exocoel. Typically in actinarians, a tentacle arises in each endocoel and exocoel.

The large ratio of surface area to body mass in sea anemones is accentuated by the numerous thin-walled tentacles that account for 50% to 80% of the external surface in various species (Shick, 1991). Tentacles are arranged around the mouth in multiples of six (Friese, 1972). The mouth is separated from the nearest tentacle ring by a smooth space known as the peristome. Where multiple rings of tentacles occur, the innermost ring represents the oldest structures.

Aiptasia pallida attaches to its chosen location at the proximal end of the polyp known as the pedal disc (figure 1.4). This attaches firmly but reversibly to the substrate with a protein-chitin cement (Shelton, 1982). Various cells in the ectoderm effect quick release of the basal disc (Shick, 1991) when necessary.

The muscular system of anemones is well developed despite being the most primitive in existence (Hyman, 1940). The major musculature in actinarians is endodermal (Shick, 1991). The epidermal muscle system is restricted to longitudinal action in the tentacles and

to the radial muscles in the oral disc. The gastrodermal muscle includes a circular layer in the tentacles, oral disc, pharynx, column wall and the pedal disc.

Cnidarians are the simplest metazoans to possess a nervous system (Shick, 1991). The nervous systems of anemone are nerve nets (diffuse two-dimensional plexuses of bi- or multipolar neurones). The complexity and integration of the nerve net found in cnidarians is surprising in view of their apparently low level of organisation. The distinctive feature of a nerve net is the way the activity spreads out in every direction from any point of stimulation. Tactile stimulation results in fast responses such as contraction or tentacle writhing. Despite a long-standing knowledge of the importance of light in the biology of anemones and their responsiveness to it, no photoreceptor structure or pigment has been found in any sea anemone (Shick, 1991).

Virtually all shallow water benthic cnidaria at low latitudes have associations with unicellular dinoflagellate algae of the genus *Symbiodinium* (Wang and Douglas, 1999), commonly referred to as zooxanthellae. Zooxanthellae are brown, coccoid cells with a diameter of 6.0-10.0 μm (Glider *et al.*, 1980) (figure 1.5). Within the host, zooxanthellae are contained by endodermal or host cells of the cnidarian. Inside the host cell, zooxanthellae are located within vacuoles (Glider *et al.*, 1980) and enclosed in a host-derived membrane known as the perisymbiont membrane (Gorian *et al.*, 1997). In *A. pallida*, zooxanthellae are found primarily within the gastrodermal tissues of tentacles and oral disc, but also occur in gastrodermal tissue of the mesenteries (Glider *et al.*, 1980). Zooxanthellae reach maximum densities of 4-6 per endodermal cell in *A. pallida* (Glider *et al.*, 1980), and are commonly concentrated in the tentacles and the oral disc where they are maximally exposed to direct sunlight over a large surface area. When host cnidarian tentacles are relaxed, gastrodermal cells are appressed tightly to the mesoglea forming a layer that lines the tentacle lumen (Glider *et al.*, 1980), the thin layer of animal tissue containing them gives little shading.

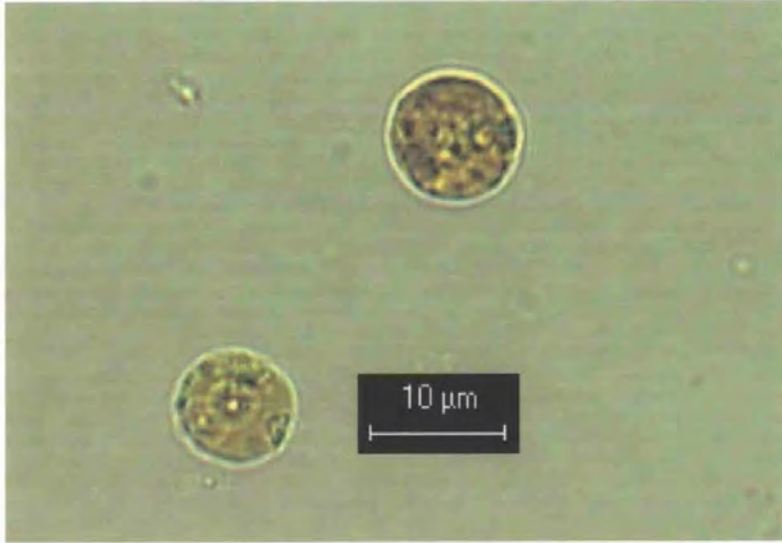


Figure 1.5. Two *Symbiodinium* spp. (zooxanthellae) isolated from *A. pallida*.

By associating with primary producers within their own bodies, zooxanthellate sea anemones become microcosms in which organic productivity and structural diversity are maintained by internal recycling of scarce nutrients, with much of the required energy coming from sunlight (Shick, 1991). The high productivity of coral reefs is due, in large part, to the photosynthesis of zooxanthellae that reside in the tissues of corals and other tropical cnidarians (Ferrier, 1992).

Zooxanthellate and azooxanthellate are terms used to distinguish cnidarians that do or do not harbour the photosynthetic associations respectively. The term aposymbiotic refers to anemones that normally have a zooxanthellae association but through natural or artificial circumstances have eliminated their symbionts.

In Bermuda *A. pallida* is associated with the symbiont *Symbiodinium* spp. (Trench, 1993). The zooxanthellae from *A. pallida* are of the 'B' restriction fragment length polymorphism genotype (as defined by Rowan and Knowlton, 1995), and are a preponderance of zooxanthellae from other cnidarians in Bermuda (Trapido-Rosenthal pers. comm.). The symbionts associated with *A. pallida* in Bermuda are indistinguishable at the 24 rRNA gene level from the symbionts from *A. pulchella* from Taiwan (pers. comm. A. Savage 1999).

A critical question in the effort to understand the nature of the symbiotic relationship between cnidaria and zooxanthellae is whether, and to what extent, the intimate associations between hosts and symbionts results from the interplay between the products of their respective genomes (Weis and Levine, 1996). Still completely un-described is the inter-partner communication and signalling that must occur both during the onset of the association, and throughout its continuing maintenance (Weis and Levine, 1996). There is evidence that zooxanthellae can influence the behaviour of the host (Pearse, 1974) and evidence supporting the concept of molecular signalling between symbionts and hosts is rapidly accumulating (Trench, 1993). Identifying the signals that trigger differential expression will begin to uncover the complex communication process in which the animal

and algal partners must engage (Weis and Levine, 1996). Two recent studies have compared proteins in aposymbiotic and symbiotic hosts, and in cultured and endosymbiotic zooxanthellae. Weis and Levine (1996) observed protein synthesis in symbiotic and aposymbiotic anemones (*Anthopleura elegantissima*) and noted marked differences in protein profiles of the two anemone types. This was interpreted as indicating either underlying differences in the regulation of gene expression or in post-translational modifications of common proteins (Weis and Levine, 1996). Major changes in the populations of proteins synthesised by cultured and endosymbiotic *Symbiodinium* sp. have been noted by Stochaj and Grossman (1997). Studies of this type indicate that the conditions within the symbiosis alter the biochemistry of both the host and the algal symbiont.

Sea anemones grow indeterminately; body size is not fixed genetically and is variable according to environmental factors that affect the balance of energy intake and maintenance costs. Sea anemone growth cannot be directly related to productivity of zooxanthellae in symbiotic associations (Muller-Parker, 1985). Zooxanthellae enhance growth only during periods of prey scarcity (Clayton and Lasker, 1984).

Anemones are carnivorous, predominately sessile feeders. The feeding behaviour of *A. pallida* has been well studied (Clayton, 1986a and 1986b). Chemical stimuli are the major cause of sea anemone feeding behaviours. Chemoreceptors are located on the tentacles, oral disc, peristome and actinopharynx (Shick 1991). Individual and population growth of *A. pallida* is significantly affected by the feeding regime (Clayton and Lasker, 1985). Zooxanthellae carry out photosynthesis within the confines of the host cell; by this means they make significant contributions to host cell metabolism by translocation of organic compounds to the host (Sutton and Hoegh-Guldberg, 1990). As such, zooxanthellae contribute to the growth and maintenance of their host (Stochaj and Grossman, 1997; Clayton, 1986a). Animal-derived nitrogenous waste compounds are recycled by the

zooxanthellae into useful compounds, especially essential amino acids, which are translocated back to the animal tissue (Wang and Douglas, 1999), thereby making the most efficient use of scarce resources (Stochaj and Grossman, 1997). By associating with zooxanthellae, cnidarian hosts gain access to two complex metabolic capabilities generally absent from the animal kingdom, namely photosynthesis and essential amino acid synthesis (Douglas, 1984). The host appears to exert strong control over the translocation of metabolites from the endosymbiont (Smith, 1939) and up to 98% of the carbon fixed by zooxanthellae is released to the host (Stochaj and Grossman, 1997). The nutritional provision by the zooxanthellae has been highlighted by a study by Balser and Fox (1998) that showed that unfed *A. pallida* associated with zooxanthellae and maintained in the light had no weight loss for at least 50 days, whilst anemones of the same type deprived use of their symbionts showed a 75% weight loss over the same period. While *A. pallida* mediates the uptake of dissolved free amino acids (Ferrier, 1992), zooxanthellae have been shown to be responsible for the uptake of ammonium into the symbiosis with *A. pallida* (Wilkerson and Muscatine, 1984). Phosphate uptake by *A. pallida* was observed only when anemones were starved and was facilitated by zooxanthellae (Muller-Parker *et al.*, 1990) indicating that inorganic nutrient fluxes are influenced by the nutritional history of the hosts (Muller-Parker *et al.*, 1990). Results of Cook *et al.*, (1988) suggest that ambient nutrient levels within the symbiosis influence the ability of the zooxanthellae to synthesise chlorophyll a.

Aiptasia pallida is a mesomyarian acontiate anemone that proliferates exclusively asexually by basal laceration (Shick, 1991). Basal laceration has a low index of reproductive effort (Hunter, 1994) and results in the formation of unisexual clones (Sterrer, 1986). Asexual reproduction by symbiotic individuals of *A. pallida* has been noted to increase biomass at a greater rate than does individual growth (Clayton and Lasker, 1985). Under normal conditions, the presence of zooxanthellae has little effect on reproductive rate; however during starvation, the presence of zooxanthellae may enhance pedal laceration (Mercier *et*

al., 1996). The use of clonal organisms to study responses to environmental factors is advantageous as variance due to genetic factors is eliminated. The ease of producing clonal populations of animals makes *A. pallida* of great value to toxicological studies.

Cnidarians have no special respiratory mechanism (Hyman, 1940) but the anthozoan body plan ensures that no cell is far removed from the surrounding medium. Diffusion is adequate to supply the necessary oxygen. The diffusion constant for anemone tissues appears to be directly related to the water content of a tissue (Shick, 1991). In response to nutritional stress (induced by an absence of food provision), host respiratory rates are reduced (Clayton and Lasker, 1984).

Anemones rely on a variety of mechanisms to avoid predation and other means of destruction. A degree of self-defence is achieved by the anemone withdrawing its tentacles into its own protective interior. Many species of anemones respond to predatory stimuli by contracting; detachments from substrate and column inflation are also noted sea anemone defensive behaviours (Edmunds *et al.*, 1976). Contraction can be provoked by any strong stimulus that might be considered unfavourable (Pearse, 1974), but expansion and contraction continue in anemones in the absence of environmental stimulation (Batham and Pantin, 1950). Anemones use the incompressible nature of the seawater contained within their coelenterons as a hydraulic fluid. By contracting with their mouths shut, the force generated by muscular contraction can be transmitted via the hydraulic fluid to the walls of the cylinder, resulting in deformation.

Anemones possess two systems of active defence: cnidoblasts and acontia. Cnidoblasts are cells containing a stinging mechanism called a cnidae or nematocyst. Nematocysts are capsules containing a coiled capillary tube, which on stimulation discharges to the exterior by turning inside out; nematocyst discharge serves to inject poison into prey. Cnidoblasts cover each tentacle and can be found on the column and over the oral disc. Acontia are threadlike extensions of the mesenteries (located in the lower part of the gastric cavity) that

are heavily loaded with nematocysts. Acontia are exposed when an anemone is damaged. Alternatively, acontia can be extruded through pores in the column wall (cinclides) when a disturbed anemone contracts.

Species of *Aiptasia* have been observed to crawl by lying sideways on the bottom and pushing their pedal disc forward, synchronous with this is contraction of the circular muscle along the upper part of the body (Friese, 1972). The gastric cavity is then inflated to anchor the pedal disc, while longitudinal muscles contract the remainder of the body and so effectively pull it up. Locomotion in this manner can move *Aiptasia* sp. up to 2 inches per hour (Friese, 1972). Species of *Aiptasia* have been noted to make slow migrations over several weeks to decrease distance to a light source, and to rotate the oral disc toward a nearby light source (Balser and Fox, 1998).

No details of the permeability of the tissues of *A. pallida* are available. Detailed studies of the temperate sea anemone *Anemonia viridis* have shown permeability to be low compared to other tissues (Benazet-Tambutte and Allemand, 1997). Permeability coefficients for *Anemonia viridis* are independent of state of contraction (Benazet-Tambutte and Allemand, 1997). These authors suggested low water permeability might represent an adaptation for maintaining a hydrostatic skeleton without significant energy expenditure (Benazet-Tambutte and Allemand, 1997).

CHAPTER TWO
GENERAL MATERIALS
AND METHODS

Chapter Two

General Materials and Methods

Except where otherwise noted, chemical stock and working solutions were made up in deionised water prepared by a Milli-Q water purification system. Glassware was cleaned, acid washed, then well rinsed and dried prior to use. Microcentrifuge tubes used were of the polypropylene type with flat caps. Liquid volumes were measured with Gilson Pipettes with autoclaved tips. Dry volumes were measured using a Sartorius Analytic balance. Geographical locations are given in longitude and latitude respectively, with degrees, minutes and seconds for each.

2.1 Specimen collection, maintenance and preparation

Anemones

Live specimens of *Aiptasia pallida* were collected from Walsingham Pond, Smiths Parish, Bermuda (32° 20 00 North, 64° 40 06 West) (figures 2.1 and 2.2). Walsingham Pond is an anchialine lake: a saline water body that has well defined subterranean connections to the sea. The pond is tidal, but the range is restricted to approximately half of that occurring in Walsingham Bay, an adjacent open water body. Walsingham Pond receives a significant input of nitrogen and phosphorous from mangrove detritus and groundwater (Cook *et al.*, 1992). The physical characteristics of Walsingham Pond are outlined in table 2.1.

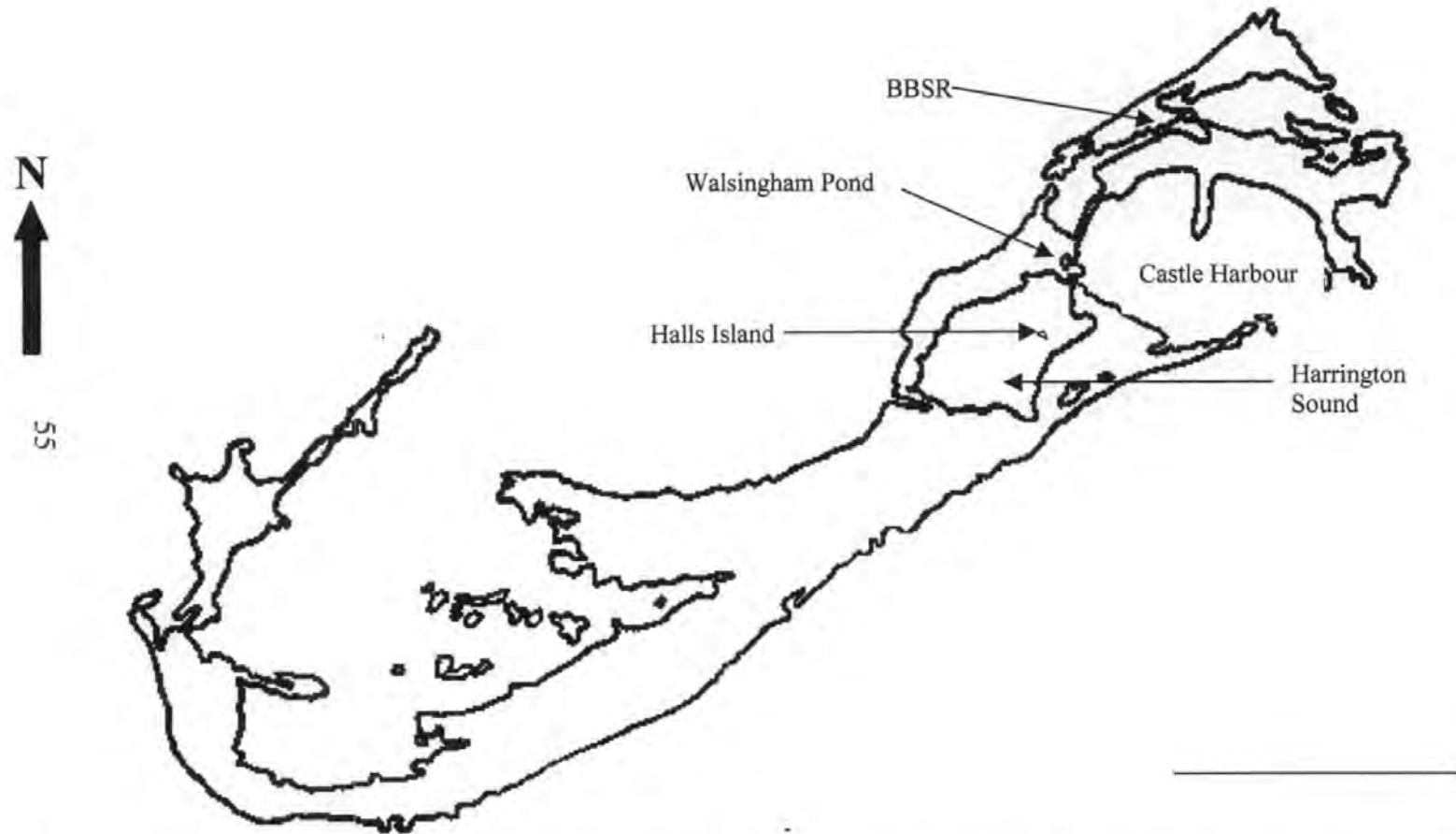


Figure 2.1. Map of the islands of Bermuda showing collection sites. *Aiptasia pallida* and *Agaricia* sp. were collected from Walsingham Pond and Halls Island respectively. The Bermuda Biological Station for Research (BBSR) was the research base used for the majority of studies documented in this thesis. Scale bar represents approximately 5 km.

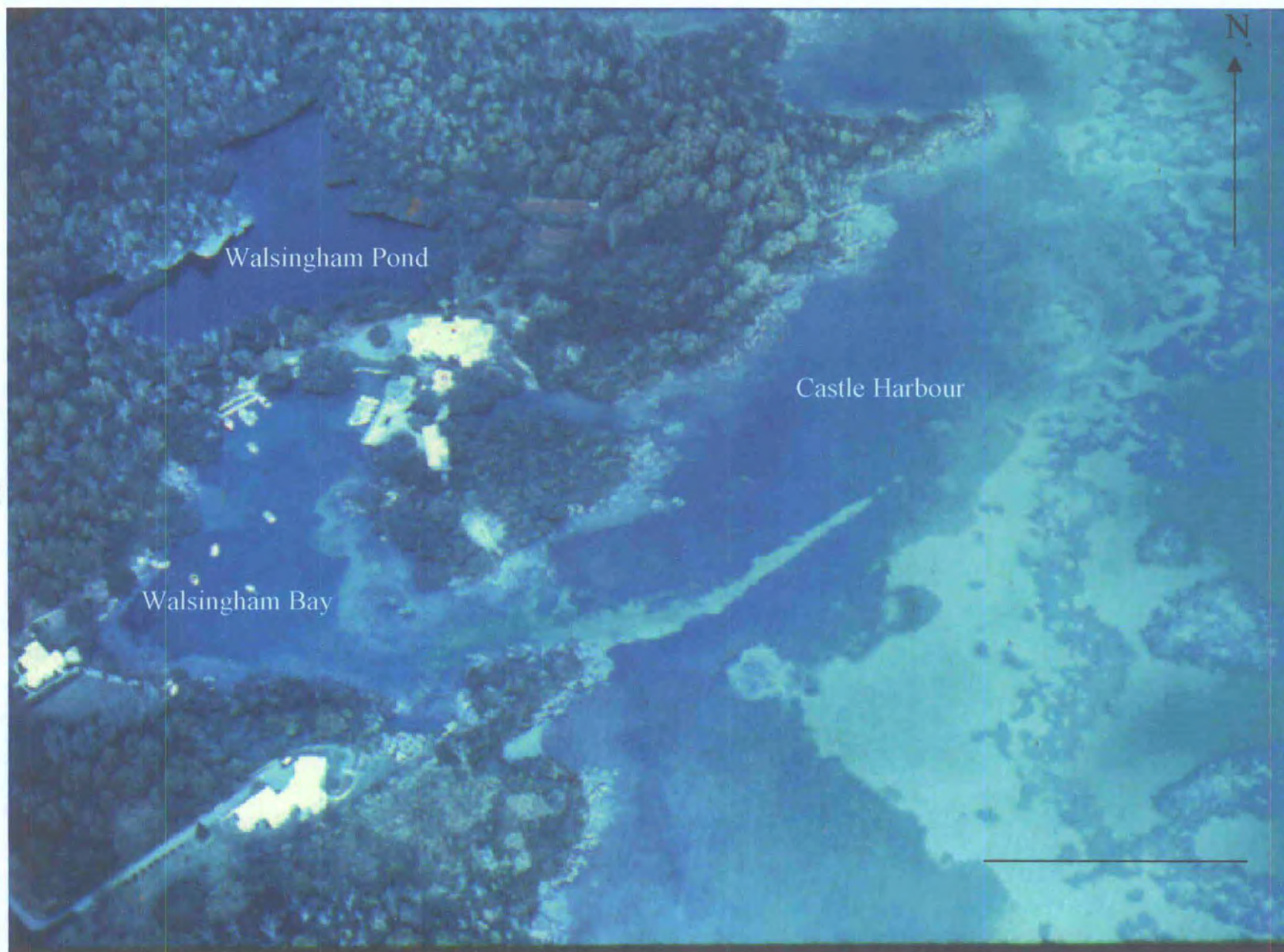


Figure 2.2. Aerial view of Walsingham Pond and surrounding area. Scale bar indicates approximately 100 m.

Area	0.79 hectare (0.08 km ²)
Volume	30,968 m ³
Mean depth	392 cm
Maximum depth	618 cm
Tidal range	38.5 cm
Maximum temperature	27.7°C
Minimum temperature	19°C
Mean temperature	23.2°C (±3.35)
Maximum salinity	39.5 ppt
Minimum salinity	33.0 ppt
Mean salinity	36.2 ppt (±1.52)

Table 2.1. Physical Characteristics of Walsingham Pond. Modified from Thomas *et al.*, 1991. Ppt abbreviates parts per thousand.

Black and red mangrove trees (*Avicennia nitida* and *Rhizophora mangle* respectively) fringe Walsingham Pond and the submerged mangrove roots provide a large surface area of hard substrate that is utilised by anemones and other invertebrates. *Aiptasia pallida* is found in abundance year round on the submerged roots of the red mangrove (figure 2.3). Walsingham Pond is approximately 4 km from the Bermuda Biological Station for Research (BBSR) where experimental work was conducted and is accessible by road or by boat (via Walsingham Bay). A round trip from BBSR to Walsingham Pond, including collection time, can be completed in less than 1 hour.

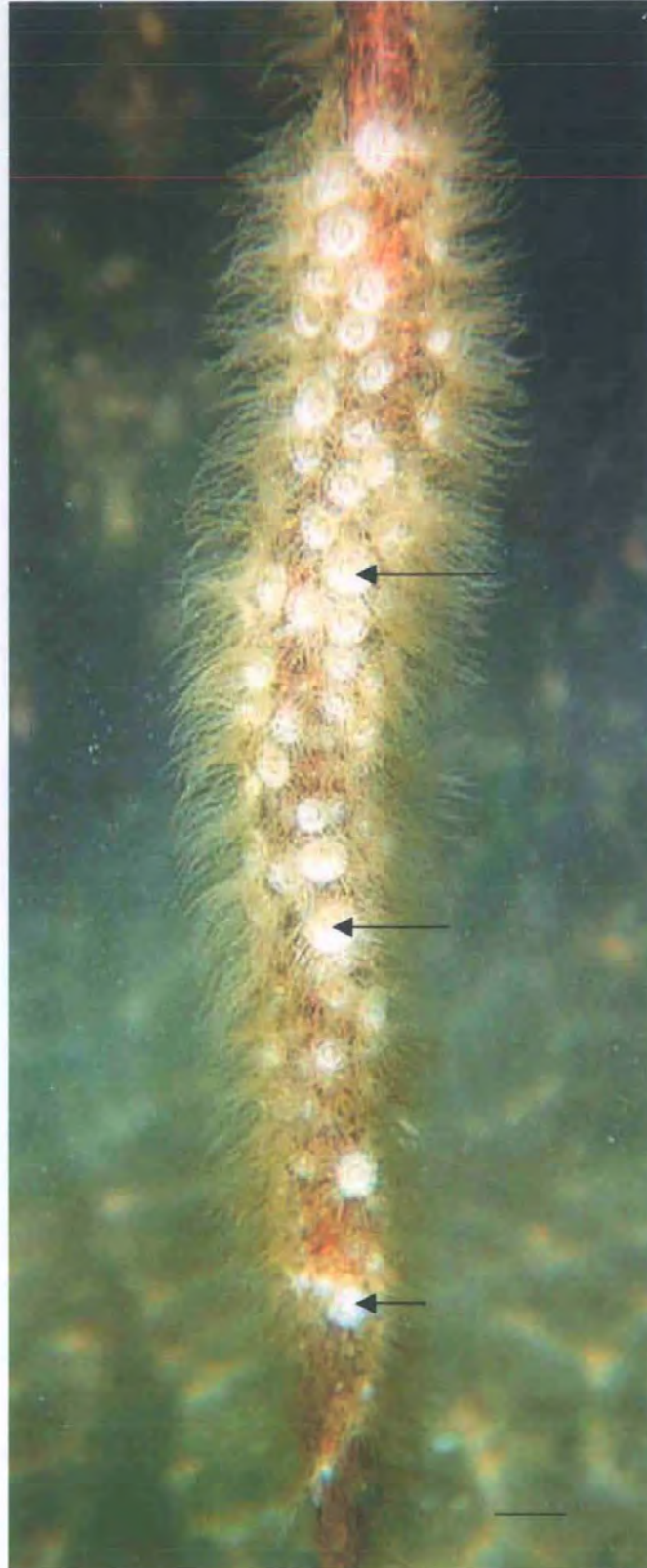


Figure 2.3. *Aiptasia pallida* on a submerged mangrove root as found at the collection site used in these studies (Walsingham Pond). Individual, white oral discs are visible (arrows point to the oral discs of selected *A. pallida*); tentacles appear as light brown strands. Scale bar indicates approximately 1 cm.

Chapter Two

Freshly collected anemones were sorted from mangrove bark and other invertebrates then transferred to clean 80 ml glass culture dishes filled with low nutrient seawater (LNSW) collected from approximately 45 miles offshore. Culture dishes were held in clear plastic boxes with lids in an incubator (Precision Scientific 818 dual program illuminated incubator) at 25°C on a 12-hour light/dark photoperiod with a light intensity of 100 μE (determined with a LiCor LI-1400 data logger using radiation sensor).

Anemones were fed twice weekly with one day old *Artemia* sp. shrimp hatched from dehydrated cysts (Argent Chemical laboratories, USA). Several hours after feeding, anemones were rinsed with fresh seawater to remove un-ingested nauplii and other debris. Anemones were transferred into clean glassware monthly to minimise algal growth.

Anemone host tissue preparation

Anemones were peeled from holding tanks at least 30 minutes prior to assay and allowed to settle in glass dishes in LNSW. The tissue level of organisation and small number of cell types present in cnidarian species (further details provided in section 1.8) makes it appropriate to use whole organism preparations in biochemical and physiological assays. Whole individuals were homogenised in a Wheaton glass homogeniser in 1 or 2 ml homogenisation buffer (HB) (50 mM HEPES, 1 mM EDTA, pH 7.4). Homogenisation was continued until a smooth, lump free liquid was obtained. This typically took 2-5 minutes. Homogenisation disrupted animal tissue but left zooxanthellae cells intact.

Post homogenisation, whole homogenate was transferred into 1.5 ml plastic microcentrifuge tubes, and then centrifuged for 10 minutes at 16000 G (Eppendorf 5415C) at 4°C. Centrifugation spun out intact zooxanthellae cells and insoluble cell material. The supernatant was purely animal in origin; microscopic examination revealed it to be free of algal cells. Unless required for zooxanthellae counts, the pellet was discarded. Supernatant, hereafter referred to as the cytosolic fraction, was then transferred into clean 1.5 ml microcentrifuge tubes and held on ice. Aliquots of the cytosolic fraction were frozen at -

20°C for protein determination. Time between sample sacrifice and running assays was minimised and was typically in the order of 15-60 minutes depending on sample number. Aposymbiotic anemones were produced by cold shocking symbiotic individuals at 4°C for 4 hours as described by Steen and Muscatine (1987). Aposymbiotic individuals were subsequently maintained in darkness at 25°C.

Anemone symbiont preparation

Zooxanthellae were separated from host material after homogenisation of whole anemones in a glass homogeniser. Suspensions of zooxanthellae in HB were sonicated for 1 minute with a Vibra Cell VC 50 sonicator (Sonics and Materials Inc. Danbury, CT, USA) to disrupt zooxanthellae cells. Biological material was maintained in plastic tubes embedded in ice jackets throughout sonication. The 50-amplitude setting was used with a 3 mm diameter probe. Centrifuge tubes were capped with Para-film to minimise liquid loss. Comparison of pre- and post sonication zooxanthellae counts enabled quantification of zooxanthellae disruption.

Corals

Harrington Sound is an inland saltwater body connected to the ocean by a narrow, shallow inlet at Flatt's Village and by submarine caves and fissures. The tidal range in the sound is reduced to about 10-15 cm compared with a 75 cm range at adjacent North Shore locations. The coral *Agaricia* sp. was collected from the Western side of Hall's island located on the eastern side of Harrington Sound (32° 20 04 north, 64° 40 02 west) (figure 2.1). Coral species were also collected from reef sites off the north shore of Bermuda. Hog Breaker (32° 27 36 north, 64° 49 48 west) is on the edge of the reef platform; Shelly Bay Shoals (32° 20 12 north, 64° 45 30 west) is a close to shore area of patch reefs (figure 2.4).

Coral samples were collected while diving on SCUBA, using hammers and chisels. Samples were placed in water filled plastic ziplock bags *in situ*, and transported back to BBSR by

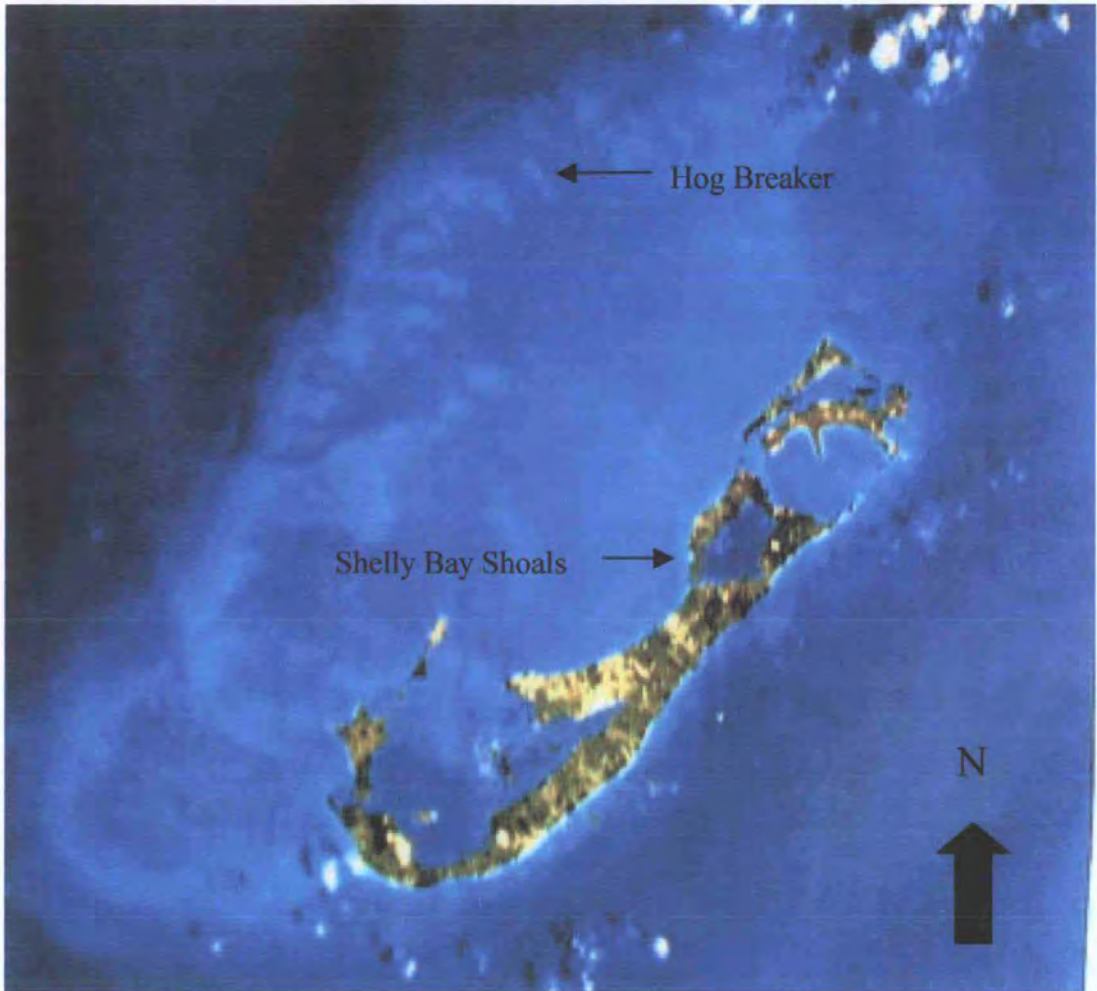


Figure 2.4. Aerial view of Bermuda (from 320 miles) illustrating two coral collection sites, Hog Breaker (where *Montastrea cavernosa* and *Montastrea franksi* were collected) and Shelly Bay shoals (where *Madracis mirabilis* and *Madracis decactis* were collected).

boat shaded in a thermally insulated box filled with water at ambient temperature. The species *Montastrea franksi* and *Montastrea cavernosa* were collected from a depth of 6 m from Hog Breaker, *Madracis mirabilis* and *Madracis decactis* from 7 m at Shelly Bay Shoals and *Agaricia* sp. from 3 m from water surrounding Halls Island. Corals were processed 1-3 hours post collection. Samples were maintained in seawater at ambient temperature prior to tissue removal.

Coral host tissue preparation

Tissue was removed from coral skeletons with a 90-psi (1 psi = 6.895 kPa) air jet from an air brush (Paasehe Type 'V', figure 2.5). A cylinder of compressed air supplied high-pressure air to the airbrush which, when applied to the coral surface, blew the coral tissue out of the skeleton. Removed tissue was collected in a plastic funnel that drained into a plastic tube. A facemask was worn to minimise inhalation of coral material. Minimal quantities of homogenisation buffer (HB) were used to maximise tissue removal whilst minimising dilution of the biological material. Aliquots of 500 µl were spread over the colony surface to encourage tissue removal. Coral tissue was homogenised in a glass Potter Homogeniser and thereafter treated in an identical manner to tissue of *A. pallida*.



Figure 2.5. Removal of coral tissue from skeleton using a Paasehe Type 'V' airbrush.

2.2 The citrulline assay

Biochemical basis and assay principle

The biochemical conversion of L-arginine to nitric oxide involves the five electron oxidation of one of the guanidino nitrogens of L-arginine to form NO coupled to the stoichiometric production of L-citrulline as shown in figure 1.1 (section 1.2). The citrulline assay is based upon detection of L-citrulline as a means of quantifying NOS activity. The citrulline assay involves addition of radioactive arginine to intact tissues or protein extracts. After incubation, assays are terminated with a buffer containing EDTA (which binds to the required calcium) and applied to columns of cation exchange resin to which the arginine binds tightly. Neutrally charged (pH 5.5) citrulline is not retained by the column and is eluted. Measurement of the radioactivity of the eluant enables quantification of citrulline production. As citrulline is generated by the activity of NOS, the activity of the enzyme can be quantified. In addition, as NO and citrulline are produced stoichiometrically, production of NO or related redox species can be inferred from measures of citrulline generation.

The citrulline assay has several advantages over other techniques: use of radioactivity makes the technique sensitive to the picomolar range; direct enzymatic conversion of arginine to citrulline in eukaryotic cells is unprecedented, so the assay is specific for the NOS pathway, and the separation of citrulline (which at pH 5.5 is of neutral charge) is facile. These advantages have resulted in the citrulline assay becoming the standard assay for NOS activity in crude and purified preparations (Bredt and Schmidt, 1997).

Materials

[^3H] arginine (38.5 Ci/mm 1424.5 GBq/mm) was obtained from New England Nuclear, USA. The remaining chemicals, including Dowex 50WX8-100 strongly acidic cation exchanger, L-arginine hydrochloride, N ω -nitro-L-arginine, N G methyl-D arginine acetate salt, and NG-methyl-L-arginine acetate salt, were obtained from Sigma USA.

The following solutions were made up at least twice monthly in ultra pure water and were used throughout the research period: homogenisation buffer ((HB), 50 mM HEPES, 1 mM EDTA, adjusted to pH 7.4), stop solution ((SS) 100 mM HEPES, 10 mM EDTA, adjusted to pH 5.5). Chemical components of a cofactor mix were made up at stock concentrations in HB as detailed in table 2.2, then stored at -20°C . Cofactor mix components were pre-mixed shortly before incubation initiation. Selected assays were conducted without addition of cofactor mix. In such cases, an arginine mixture was used to initiate the reaction. A total of 1.1 μl premixed unlabeled L-arginine and labelled [^3H] L-arginine (ratio 0.6:0.5 v/v respectively) arginine mix was added to each incubation. Final concentration of L-arginine was 20 μM (6000 pmoles from L-arginine, 12.5 pmoles from [^3H] L-arginine). Typically triplicate incubations of each sample were set up and assayed.

Dowex ion exchange resin was used to separate radiolabeled arginine and citrulline. Prior to use, conversion from the acidic (hydrogen) ionic form to a basic sodium form was necessary. Conversion was achieved by rinsing the resin repeatedly with deionised water, then immersing the resin in 1 M sodium hydroxide until pH of >12 was achieved. Resin was then washed in deionised water, immersed in SS, adjusted to pH 5.5 and stored at 4°C . Prior to use the pH was checked and adjusted to 5.5 when necessary. Filtration tubes (3 ml) fitted with polyethylene frits were set up in 15 ml plastic centrifuge tubes.

Chemical	Stock concentration	Volume per incubation (μl)	Final concentration in incubation volume (300 μl)
NADPH	15 mM	20	1 mM
CaCl₂	25 mM	15	125 mM
DTT	60 mM	5	1 mM
BH₄	20 mM	0.15	10 μ M
Calmodulin	50 units/ μ l	3	150 units
L-arginine (unlabeled)	10 mM	0.6	20 μ M
[³H] L-arginine	1 m Ci/ ml	0.5	0.042 μ M
Homogenisation buffer	50 mM HEPES/ 1 mM EDTA	55.75	9.29 mM/ 18.5 μ M

Table 2.2. Cofactor mix details: stock concentrations of components, volume of stock added to each incubation and the final concentration in the whole incubation volume.

Ion exchange resin (1 ml) was added to the filtration tubes just prior to assay. Resin columns were rinsed twice with 2 ml stop solution. Resin was maintained moist by the addition of 500 μ l stop solution.

The assay

Principal steps

1. Incubation of biological material with radioactive arginine.
2. Ion exchange chromatography to separate L-arginine from L-citrulline.
3. Liquid scintillation counting of L-citrulline.

Chapter Two

Incubations were conducted in 1.5 ml plastic microcentrifuge tubes at 25°C. Biological material was prepared as detailed in section 2.1. Typically 10-100 µl cytosol was used. Volumes of HB were adjusted in order to maintain a total incubation volume of 300 µl throughout. Where applicable, 100 µl cofactor mix was added to initiate the incubation. Incubations without reaction cofactor mix were initiated by addition of 1.1 µl arginine mix. Incubation vessels were capped and pulse vortexed immediately after the addition of cofactor mix or arginine mix. Blanks were equivalent to incubation conditions of the experiment with biological material excluded.

Incubations were quenched after 30 minutes by the addition of 900 µl SS to each incubation. Entire incubation volumes were then put onto the ion exchange columns. Force was applied to the columns to speed the movement of liquid through the resin (pulse centrifugation on a desktop international clinical centrifuge). Columns were rinsed twice with 1.5 ml deionised water. Eluant (total volume 4.2 ml) was then mixed and a 1 ml aliquot taken for liquid scintillation counting. Background radioactivity was quantified with aliquots of cofactor mix or arginine mix (5 µl or 0.5 µl respectively) in 5 ml of Ultima Gold LSC cocktail. Liquid scintillation chromatography was conducted in 7 ml glass vials (USA Scientific) and was facilitated by addition of 5 ml of Ultima Gold. Scintillation vials were thoroughly mixed then counted by a Packard Tri-Carb 4530 liquid scintillation counter. Each count cycle ran for a 10-minute period.

Data analysis

Disintegrations per minute (DPM) were converted to picomoles citrulline and expressed per µg protein per minute incubation. Values were blank corrected. Background counts were used to calculate the specific activity of the exogenous arginine. Protein content of cytosolic preparations was determined as detailed in section 2.6. Calculations were performed using a

Microsoft Excel spreadsheet. T-tests and Kruskal-Wallis statistical tests were used to determine the statistical significance of obtained results.

2.3 The FRAP assay

Biochemical basis and assay principle

The ferric reducing/antioxidant power (FRAP) assay is a recently described measure of 'total antioxidant power' (Benzie and Strain, 1996, 1999). A biological antioxidant can be defined as any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Benzie and Strain, 1999). The FRAP assay principle is that antioxidants in the sample reduce ferric (FeII) 2,4,6-tripyridyl-s-triazine (TPTZ) to ferrous (FeIII) TPTZ which has an intense blue colour with a strong absorbance at 593 nm. Absorbance changes are directly related to the total reducing power of the electron donating antioxidants present in the reaction mixture. Total antioxidant capacity is therefore analogous to total reducing power. In comparison to other methods for assessing antioxidant capacity (reviewed by Prior and Cao, 1999) the FRAP assay does not require specialised equipment and is simple, speedy, inexpensive, and robust (Benzie and Strain, 1999).

Materials

Glacial acetic acid was obtained from the Fisher Scientific Company, USA; 2,4,6, - tripyridyl-s-triazine (TBTZ) and all other chemicals were obtained from Sigma USA.

The FRAP reagent was made up by mixing three solutions, each made up in deionised water and stored in glass capped vessels at 4°C. FRAP reagent was prepared fresh as required by mixing solutions 1, 2 and 3 in the ratio 10:1:1 respectively. Reagents and samples were allowed to reach room temperature prior to use. Solution 1: 300 mM acetate buffer in 1.6 % glacial acetic acid adjusted to pH 3.6. Solution 2: 10 mM TBTZ and 40 mM HCl. Solution

3: 20 mM FeCl₃. 6 H₂O. Iron sulphate standard (FeSO₄) was made up from a stock of 1000 μM FeSO₄ made up in deionised water.

The assay

Principal steps

1. Biological material mixed with FRAP reagent.
2. Absorbance of the sample and FRAP mix monitored over 6 minutes.

Standards of FeSO₄ in the range 100-1000 μM were run prior to each set of sample assays. Cytosolic samples were assayed immediately after preparation of the biological material (section 2.1). Samples or standards (20 μl) were well mixed with 600 μl FRAP reagent. Absorbances were measured immediately and monitored for 3 or 6 minutes for standards and samples respectively at 593 nm using a Spectronic Genesys 5 spectrophotometer (these reaction times were used to encompass the uric acid, α-tocopherol, ascorbic acid, and bilirubin-related absorbance changes (Benzie and Strain, 1996)). Blanks were prepared for each sample with FRAP reagent alone. Fresh FRAP reagent was made up each 30 minutes and used at room temperature (25°C).

Data analysis

FRAP values were calculated by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (Benzie and Strain, 1996) using equation 2.1:

$$\frac{\Delta \text{ abs. sample } 0 - 4 \text{ min @ } 593\text{nm}}{\bar{x} \text{ abs. } \Delta \text{ std @ } 593\text{nm}} \times \text{FRAP value of std } (\mu\text{M}) \quad \text{Equation 2.1.}$$

The blank corrected signal given by a 100 μM solution of Fe (II) is equivalent to a FRAP value of 100 μM (Benzie and Strain, 1999).

2.4 The neutral red assay

Biochemical basis and assay principle

The Neutral Red Assay technique utilises live cells to assess lysosomal destabilisation (Ringwood *et al.*, 1998) and enables the construction of lysosomal destabilisation indices. Lysosomal destabilisation has been identified as a biomarker of general stress, and a wide range of marine species have been evaluated with the technique. Neutral red (NR) is a cationic probe (Lowe *et al.*, 1995a). The lipophilic nature of NR results in it preferentially accumulating in lysosomes, where it is trapped by protonization (Ringwood *et al.*, 1998). Stable lysosomes retain dye for extended periods (several hours) whilst damaged cell membranes leak NR within a short time frame. Observation of NR retention over time enables quantification of the leakage of lysosomal contents into the cytosol that occurs when membranes are damaged (Lowe *et al.*, 1992). Quantitative assessment of NR retention can as a result be used as an indicator of the health of lysosomal structures, which can be extrapolated and used as a measure of whole organism health.

Lysosomes

Lysosomes are small (0.25–0.5 μm in diameter) round organelles found within almost all cells. They are membrane bound, dense bodies that contain a number of hydrolytic enzymes that are active at acid pH. Lysosomes represent the digestive system of the cell, and as such they are involved in numerous functions, including nutrition, tissue repair, cellular defence and turnover of membranes, organelles, and proteins. Lysosomes have a remarkable ability to accumulate a diverse range of toxic metals and organic chemicals (Moore, 1990) and there is a substantial body of literature implicating environmental pollutants, organic xenobiotics and metals in lysosomal destabilisation in fish and molluscs (Ringwood *et al.*, 1998). The lysosomal membrane serves as a protective physical barrier between the lysosomal hydrolytic enzymes and the rest of the cell; lysosomal membrane integrity is

therefore critically important to the cell and leakage of lytic enzymes into the cytosolic compartment can result in autogenous degradation of cellular macromolecules and other components (Winston *et al.*, 1991). Cytochemical investigations have shown that chemical contaminants induce profound structural and functional alterations in lysosomes (Ringwood *et al.*, 1998).

Materials

Neutral red (C.I. 50040; 3-Amino-7-dimethylamino-2-methylphenazine hydrochloride), trypsin (TPCK type) and dimethyl sulphoxide (DMSO) were obtained from Sigma, USA.

The following solutions were made up in deionised water and used in the preparation of biological material for assay: physiological saline (PS) (0.2 M HEPES, 4.4 M sodium chloride, 0.5 M magnesium sulphate, 0.1 M potassium chloride and 0.1 M calcium chloride, pH adjusted to match ambient seawater), calcium-magnesium free saline (CMFS) (0.02 M HEPES, 0.5 M NaCl, 0.01 M KCl, 0.005 M EDTA, gassed for 10 minutes with 95% oxygen, then adjusted to pH 7.3) and trypsin solution, (416.7 mg/l). Homogenisation buffer (HB) was prepared as detailed in section 2.1. Stocks of 0.1 M NR were prepared in DMSO. Gentle agitation of the suspension ensured dissolution of NR. Stock NR was filtered through a 0.5 μ M Millipore filter attached to a hypodermic syringe (both sterile packed) then stored in a smoked amber vial within a light proof container at 4°C. Working solutions of 5 mM NR were prepared immediately prior to use, with well-mixed room temperature NR stock and physiological saline. Working solutions were stored temporarily in smoked amber vials in darkness. Poly-L-lysine working solution was made up fresh prior to each use by diluting the manufactured product 1 in 10 with deionised water (Poly-L-lysine increases adhesion of cells onto the slide surface by altering the charge of the cell coat).

The assay

Principal steps

1. Preparation of biological material
2. Biological samples incubated with NR.
3. Observation and monitoring of stained cells.

Preparation of biological material

The neutral red assay was run initially under instruction (Miss. V. Cheung, University of Plymouth) with haemolymph extracted from the mussel *Mytilus edulis* and clear lysosomes were visualised, (illustrated in figure 2.6). Use of the neutral red assay has not been previously reported in cnidarian species. Techniques previously documented for use with a range of both vertebrate and invertebrate preparations (Lowe and Pipe, 1994; Grundy *et al.*, 1996; Lowe *et al.*, 1995a; Lowe *et al.*, 1992) were modified for use with *A. pallida*. A number of approaches were used to prepare *A. pallida* for the neutral red assay. Preparations were performed at 25°C.

Removal of haemolymph equivalent

Anemones were pierced with a hypodermic syringe and fluid was drawn from the region of the mesenteries. Fluid obtained was immediately diluted 1:1 in PS and expelled into clean microcentrifuge tubes.

Tissue disruption

Whole anemones were homogenised in glass Potter homogeniser in HB, PS or LNSW. Homogenisation was brief (5-10 seconds) and just sufficient to disrupt anemone tissues. Homogenate was centrifuged (325 RCF for 2 minutes) and both supernatant and resuspended pellet (in HB, PS or LNSW) were examined. Silk meshes (45-200 µM) were used in some preparations, both filtrate and material gathered from the filter was transferred into clean microcentrifuge tubes, and then examined microscopically.

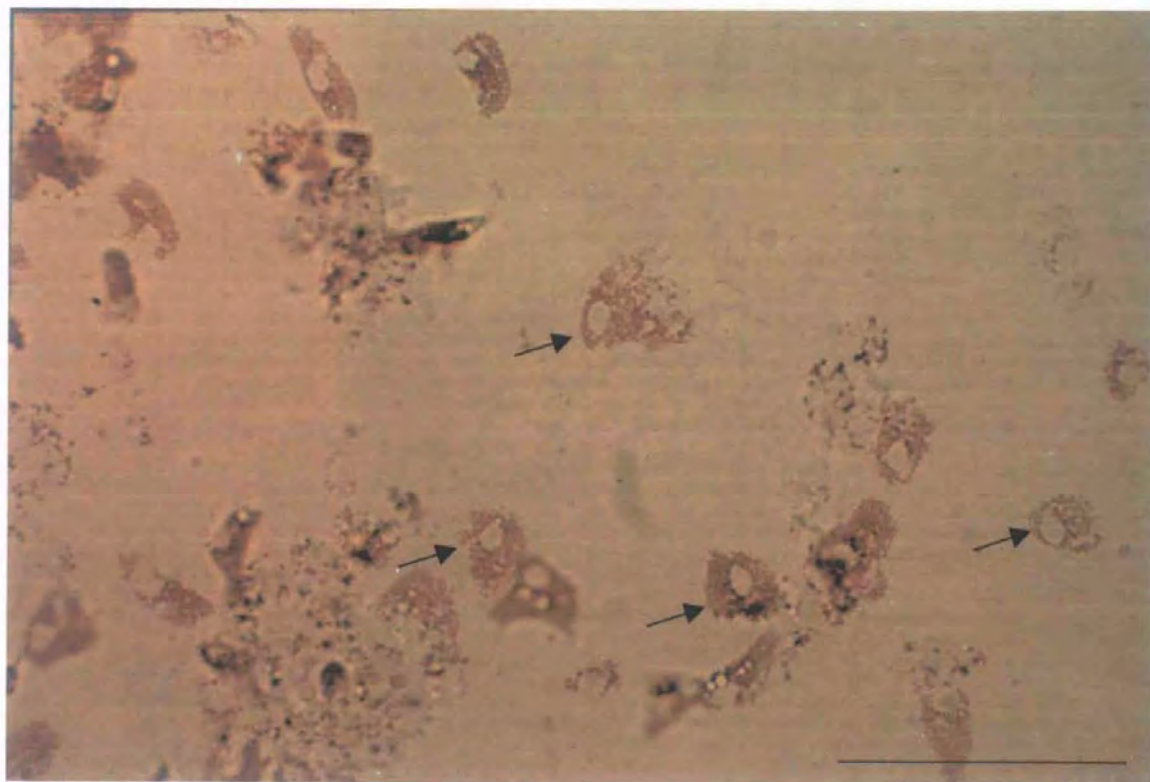


Figure 2.6. Haemolymph from *Mytilus edulis* stained with neutral red. Isolated lysosomes are indicated by arrows. Scale bar represents approximately 1 mm.

Digestion

Whole tissue digestions were carried out with trypsin on recommendation from Dr. David Lowe (from Plymouth Marine Laboratories) and after consultation of methods of Lowe *et al.*, (1992). Tissue sections were dissected from anemones, cut into small pieces (approximately 2 mm cubes), suspended in CMFS and agitated for 30 minutes in glass vials. Trypsin solution was then added and the suspension agitated for a further 30 minutes to encourage cell disaggregation. The suspension was then centrifuged (325 RCF for 2 minutes) and the cell-containing pellet resuspended in CMFS in microcentrifuge tubes.

Incubation with NR

Cell suspensions were prepared as outlined above. Glass microscope slides were smeared with 1 μ l Poly-L-lysine working solution using a coverslip, and then allowed to air dry. Biological preparations (40 μ l) were applied to slides, which were then incubated for 30 minutes in a lightproof humidity chamber at room temperature to allow cells to attach. Excess suspension was then removed from the slide and the attached cells were treated with 40 μ l NR working solution. Neutral red solution was applied by slowly ejecting the dye from a pipette tip onto the cells. After 15 minutes incubation, slides were covered with a 22 mm² coverslip, resulting in a dye concentration of <50 μ g ml⁻¹ for the incubation. Slides were then examined with a light microscope (Olympus model CH30RF100). A total of 50 cells were counted for each preparation. Slides were examined at 15-minute intervals in the first hour, then every 30 minutes. Between examination intervals slides were held in a lightproof humidity chamber at room temperature to minimise light and temperature fluctuation exposure. Condition of both cells and their lysosomal compartments was assessed and noted at each time interval. Retention time of the NR probe was assessed by scoring the percentage of cells exhibiting dye leakage from the lysosomes. Cells were also examined for changes in structure detailed in table 2.3.

Criteria	Healthy cells	Stressed cells
Cell shape	irregular	rounder
Cell size	large	smaller
Number of lysosomes	many	less
Size of lysosomes	small	enlarged
Colour of lysosomes	pale red/pink	darker red/pink/brown/orange
Pseudopodia	not seen	present
Dye leakage	not seen	maybe present

Table 2.3. Cell structural changes of note during incubation with NR.

Slides were categorised into one of the following classes: no sign of stress, some haemocytes expressing lysosomal leakage of neutral red probe, or $\geq 50\%$ cells exhibiting leaking lysosomes (NR distributed through the cytosol). When $\geq 50\%$ of cells exhibited leakage the examination of the slide was halted.

2.5 Protein determination assay

Biochemical basis and assay principle

Bradford Reagent was used to establish a protein standard curve using Bovine Serum Albumin (BSA). A regression equation was derived and used to determine the protein content of anemone and coral samples from their respective absorbance profile. Bradford Reagent contains Coomassie Brilliant Blue, a dye that exists in two colour forms, red and blue (Bradford, 1976). The red form is converted to the blue form upon binding to protein. This causes a shift in the dye absorption maximum from 465 to 595 nm. The amount of absorption produced at 595 nm is proportional to the protein concentration. The protein-dye

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complex has a high extinction coefficient leading to great sensitivity in measurement of the protein (Bradford, 1976). The dye-binding assay is reported to be approximately four times more sensitive than the Lowry assay (Bradford, 1976).

Materials

The following were obtained from Sigma, USA: Bradford Reagent and Bovine Serum Albumin (BSA). A Spectronic Genesys 5 Spectrophotometer was used to read absorbances. A stock of 25 $\mu\text{g/ml}$ BSA was prepared in homogenisation buffer and was frozen at -20°C between assays. New stock was made up monthly.

The assay

Principal steps

1. Preparation of biological material.
2. Addition of Bradford reagent.
3. Absorbance determination at 595 nm.

Protein contents of thawed, re-homogenised cytosolic samples were determined with Bradford Reagent, using BSA as standard. Duplicate protein standards in the range 0- 20 μg per cuvette were prepared as shown in table 2.5. Blanks were prepared with HB alone.

μl 25 $\mu\text{g/ml}$ BSA	μl HB	μg Protein per tube
0 (blank)	800	0
160	640	4
320	480	8
480	320	12
640	160	16
800	0	20

Table 2.4. Summary of details for protein standard curve set-up.

Aliquots of 200 μ l Bradford reagent were added to each cuvette. Blank absorbances were used to zero the spectrophotometer. Dye to protein binding is a rapid process and the protein-dye complex remains dispersed in solution for approximately 1 hour (Bradford, 1976). As a result, absorbance readings were made after thorough mixing, 5-60 minutes after the addition of Bradford Reagent. Sample volumes were adjusted to yield absorbances within the standard range (typically 0.05-0.35 at 595 nm); HB was used when dilution was necessary. Typically 5-20 μ l of homogenised sample was used; volumes of HB were adjusted to maintain a constant reaction volume of 800 μ l.

Data analysis

Mean absorbances of BSA standards were plotted against their protein content. Linear regression equations were obtained from plotting a regression line to this plot. Protein contents of samples were calculated with use of the regression equation.

2.6 Visual response monitoring

Physiological parameter measurement

Selected dimensions of anemones were measured with callipers (Manostat mechanic Type 6911). The total width of the anemone was measured (from tentacle tip to opposite tentacle tip), along with the oral disc diameter (represented by the area within the innermost tentacle ring (figure 2.7a and b). Callipers were adjusted close to, but just clear of the anemones body to avoid causing contraction.

The status of anemones was described before and during exposure to a variety of chemicals or thermal regimes. Behaviours that were particularly noted were: degree of expansion or contraction, attachment or detachment of the pedal disc, and flaccidity or turgidity of the animals. Anemones were considered dead when unresponsive to tactile stimulation. In some cases photographs were taken to assist the visual monitoring of anemones.



Figure 2.7 a and b. Illustration of axis of measurement of oral disc diameter (2.7a) and total diameter (2.7b).

2.7 Zooxanthellae density determination

Zooxanthellae are unicellular dinoflagellate algae found commonly in symbiotic association with cnidarian hosts. Zooxanthellae are located within cnidarian host cells that are located in the endodermal layer (Shick, 1991). Zooxanthellae have a robust cell membrane that is not disrupted by the techniques applied to prepare host tissues (homogenisation of entire symbiotic assemblage in glass (detailed in section 2.1)). Zooxanthellae density monitoring enabled anemone bleaching to be identified and quantified.

Materials

A solution of 95% ethanol was prepared. A haemocytometer (Improved Neubauer) was used to count the zooxanthellae aided by use of a Nikon YS2-T compound light microscope and a Compass tally register.

Zooxanthellae counting

Principal steps

1. Zooxanthellae preparation and isolation.
2. Zooxanthellae counting on haemocytometer grids

Frozen aliquots of raw homogenate were defrosted then mixed well with 1 ml of HB. Samples were thoroughly vortexed then centrifuged at 735 G (Eppendorf Centrifuge 5415C) for 3 minutes. Supernatant was then discarded and the pellet resuspended with a disposable plastic homogeniser unit (USA/Scientific Plastics) in 1 ml of HB before the sample was respun at the same settings as before. This was repeated 3 or 4 times until the samples were free of host tissue (confirmed by microscope examination). The resulting pellet was resuspended in HB to give an approximate density of 100 cells per grid. A 25 mm x 20 mm haemocytometer coverslip was applied to the haemocytometer. As the volume of liquid held on the haemocytometer grid was crucial to maintain the accuracy of the zooxanthellae count, the edges of the cover slip were moistened to assist adhesion to the haemocytometer.

Formation of Newton's rings confirmed a secure seal. Approximately 12 μ l of resuspended zooxanthellae was applied to the edge of the coverslip just above the grid. Liquid was drawn under the coverslip by capillary action. Haemocytometers were set-up on a light microscope and visualised at x40 magnification on a (Nikon YS2-T compound light microscope). Zooxanthellae numbers were determined by replicate counts (n=4-6). Coverslips and haemocytometer grids were cleaned thoroughly with 95% ethanol between samples.

Data analysis

Mean zooxanthellae counts per grid were converted into densities of zooxanthellae per volume of material. Using resuspension volume and total homogenisation volume values, zooxanthellae per anemone were calculated.

2.8 Chlorophyll determination

Principle

Zooxanthellae contain chlorophyll a and c₂. Cnidarian bleaching is defined as the loss of zooxanthellae or their pigments. To fully characterise changes induced in zooxanthellae exposed to water above ambient temperatures, it was appropriate to quantify their chlorophyll content.

Materials

Homogenisation buffer (HB) was prepared as detailed in section 2.2. Pure acetone was chilled prior to use at -20°C. Whatman GF/A glass microfibre filters (2.5 cm diameter, retention 1.6 μ M) were obtained from Sigma USA.

The assay

Principal steps

1. Zooxanthellae isolation.
2. Chlorophyll extraction.

Pellets resulting from centrifugation of whole anemone homogenate were resuspended in 600 µl HB. Resuspension was aided by use of a plastic homogeniser and thorough vortexing. Resuspended homogenate was pipetted onto a filter set-up on a Buchner vacuum-filter apparatus. Filters were washed with a further 600 µl HB. Once dry, filters were removed with forceps and ground in a pestle and mortar (pre chilled at -20°C) with 2 ml chilled acetone. Control filters (free of biological material) were washed with HB then ground and treated in an identical manner to the samples from this point on.

Homogenised filters were transferred to 2 x 1.5 ml plastic microcentrifuge tubes. The pestle and mortar was rinsed with 1 ml cold acetone and the rinse solution added to the microcentrifuge tubes in an even manner. Tubes containing filter material and acetone were wrapped in foil and incubated for three hours at 4°C. After this incubation, samples were centrifuged at 4°C for 10 minutes at 16,000 G. Supernatant was transferred into labelled glass bottles wrapped in foil. A further 400 µl acetone was added to each pellet. Pellets were resuspended (by vortexing) then incubated in the dark overnight at 4°C. Comparison of sample pellets with the control pellet gave an indication of the chlorophyll remaining to be extracted. After overnight incubation and approximately 24 hours after initial acetone addition, filter homogenate extractions were spun down (10 minutes at 16,000 G) and the resulting supernatant added to the appropriate vial. Total extraction volumes were determined at this stage for use in future calculations. Aliquots of 1200 µl of each extraction were transferred to clean microcentrifuge tubes and spun for 10 minutes at 16,000 G at 4°C to pellet any residual glass fibres. Absorbance of the resulting supernatant was measured at

630 nm and 663 nm in glass cuvettes. Blanks of 1 ml acetone removed from the control filter were used to zero the spectrophotometer.

Chlorophyll concentration calculation

Chlorophyll a and c_2 concentrations were calculated with the equations of Jeffery and Humphrey (1975), equations 2.2 and 2.3:

$$\text{Chlorophyll a (in } \mu\text{g/ml)} = (11.43 \times \text{absorbance at 663 nm}) \times (0.64 \times \text{absorbance 630 nm})$$

Equation 2.2

$$\text{Chlorophyll } c_2 \text{ (in } \mu\text{g/ml)} = (27.09 \times \text{absorbance 630 nm}) \times (3.63 \times \text{absorbance. 663 nm})$$

Equation 2.3

Using measurements of total amount of acetone used in the extraction, the concentration of chlorophyll in the entire extraction was calculated. Chlorophyll concentrations were related to μg host protein and to zooxanthellae cell counts.

2.9 Statistical methods

Excel spreadsheets were used for basic calculations (to derive mean, standard deviation, coefficient of variation and linear regression values) and for conducting student t-tests. Statgraphics software was used to complete analysis of variance (ANOVA) and Kruskal-Wallis statistical analyses to test for significant differences among treatments. Graphpad PRISM software was used to determine the kinetic parameters of the NOS enzyme. Enzyme substrate kinetics (V_{max} and K_m) were determined from a non-linear regression of data. K_i was determined with the equation of Cheng and Prusoff (1973).

CHAPTER THREE
CHARACTERISATION
OF NITRIC OXIDE
SYNTHASE

Chapter Three

Characterisation of Nitric Oxide Synthase

3.1 Introduction, rationale, materials and methods, and results

The presence of NOS activity was investigated in cytosolic preparations of *A. pallida* and a variety of coral species. Basal rates of NOS activity were determined in these species and in the dinoflagellate symbiont associated with *A. pallida*, *Symbiodinium* spp.. A range of citrulline assay parameters were tested to determine the suitability of the protocol for use with cnidarian tissues. Following identification of NOS enzyme activity in *A. pallida*, experiments were designed to characterise the enzyme in terms of parameters described for other NOS enzymes, including substrate kinetics, cofactor requirements and responses to exposure to arginine analogues previously described as inhibitors of isoforms of NOS. This enabled comparison of the characteristics with other previously investigated NOS isoforms. Regrettably, only after completion of experimental work was it appreciated that the citrulline assay used in these studies was not optimised for use with *A. pallida* preparations. Consequently the accuracy of the results derived from these assays is uncertain. The recognized problems, along with their implications are detailed in this chapter's discussion.

Owing to the diverse characterisation research conducted, specific rationales, methodologies and results for each of the components of this research are presented consecutively.

Assay validation

NOS activities were measured using the citrulline assay (section 2.2). Routine use of the Greiss assay (based on the methods of Tracey, 1992) was found to be impractical owing to the limited instrumentation available and an apparent lack of assay sensitivity.

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Determination of the protein content of biological samples enabled rates of citrulline formation to be expressed as pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$; protein concentrations were determined by means of the Bradford assay (Bradford, 1976) (section 2.5). Results shown are means of triplicate determinations \pm standard deviation (s.d.). Except where otherwise stated, the standard citrulline assay protocol (detailed in section 2.2) was used with freshly prepared cytosolic samples from long term incubator maintained *A. pallida* incubated with 20 μM exogenous L-arginine. Quantification of rates of citrulline formation enabled indirect quantification of NO formation and hence NOS activity. Specific rationale, protocol and results for each of the characterisation steps are detailed below.

Intra assay variance

Rates of citrulline formation in 18 identical aliquots from a single anemone preparation were determined by standard citrulline assay in order to quantify levels of intra assay variance.

Results

A mean value of 0.88 ± 0.04 pmoles citrulline formed per $\mu\text{g protein}^{-1} \text{ min}^{-1}$ was calculated from the combined data, with a coefficient of variation equal to 4.17%.

Eluant acceleration

Incubation volumes were accelerated through ion exchange columns by pulse centrifugation. As this represented a modification from standard published protocols, the effect on determined levels of citrulline activity was investigated. The standard citrulline assay was run using aliquots of cytosol from a single anemone preparation up to addition of incubation volumes to the ion exchange columns. Eluant was then either accelerated by

pulse centrifugation or eluted by gravity flow for one or three hours. Rates of citrulline formation in each eluant were determined and compared.

Results

Similar rates of citrulline generation (pmoles citrulline formed per $\mu\text{g protein}^{-1} \text{ min}^{-1}$) were detected in the centrifugally accelerated eluant (1.34 ± 0.03 , $n=3$), and the eluant allowed to flow through ion exchange columns over either one (1.33 ± 0.01 , $n=3$) or three hours (1.27 ± 0.02 , $n=3$).

Endogenous L-arginine

It has been previously noted that owing to the presence of variable amounts of L-arginine, whole tissue assays may not provide reliable measurements of NOS activity (Giraldez and Zweier, 1998). Endogenous L-arginine will dilute labelled substrate, and potentially reduce the detected citrulline formation, leading to an underestimation of NOS activity (Giraldez and Zweier, 1998). Investigations were conducted to determine the influence of endogenous arginine on rates of citrulline formation detected by the citrulline assay. Endogenous arginine was removed prior to incubation by eluting cytosol through a Dowex ion exchange column (set up as for a standard citrulline assay) with homogenisation buffer (as previously documented by Radomski *et al.*, 1991) and rates of citrulline formation compared with standard cytosolic preparations. Incubations were initiated by addition of standard arginine mix.

Results

No difference in rates of NOS activity were observed between untreated samples and samples from which any existing endogenous arginine had been removed; rates of citrulline generation were determined to be 3.08 ± 0.09 and 3.07 ± 0.09 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ respectively.

Incubation with L-valine or L-ornithine

L-arginine may be indirectly converted to L-citrulline by arginine and ornithine transcarbamylase (Giraldez and Zweier, 1998). This is a two-step reaction in which the enzyme arginase catalyses the formation of urea and L-ornithine; the latter substance is then converted to L-citrulline by ornithine transcarbamylase (Giraldez and Zweier, 1998). The arginase inhibitors L-valine (1 mM) and L-ornithine (1 or 1.5 mM) (Knowles and Moncada, 1994; Giraldez and Zweier, 1998) were co-incubated with cytosolic preparations in standard assays to investigate involvement of arginase. Incubations were initiated with either cofactor mix or arginine mix. Control cytosol was incubated without arginase inhibitors.

Results

Rates of citrulline formation in cytosolic samples incubated with L-valine or L-ornithine were unchanged compared to untreated controls as shown in table 3.1.

Final concentration/ chemical added	Control (pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation)	Treated (pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation)
* 1 mM L-valine	1.22 ± 0.14	1.21 ± 0.07
1 mM L-valine	0.45 ± 0.05	0.43 ± 0.02
<u>1 mM L-valine</u>	<u>1.20 ± 0.01</u>	<u>1.19 ± 0.67</u>
<u>1 mM L-ornithine</u>	<u>1.20 ± 0.01</u>	<u>1.24 ± 0.04</u>
*1.5 mM L-ornithine	0.87 ± 0.002	0.95 ± 0.01

Table 3.1. Rates of citrulline formation in cytosolic preparations incubated with (treated) or without (control) L-valine or L-ornithine. Values shown are means of triplicate counts \pm s.d.. Rows, except those that are underlined, represent data from separate experiments. Asterisks denote incubations to which cofactor mix was added.

Enzyme stability

Temperature stability

Aliquots of cytosol from a single preparation were maintained for two hours at 0°C, -20°C, -70°C or 100°C to investigate the stability of the NOS enzyme in standard *A. pallida* cytosolic preparations. Sample aliquots were contained within microcentrifuge tubes during exposure to these thermal regimes. Frozen samples were defrosted and all samples were at room temperature when assayed.

Results

Citrulline activity in boiled aliquots was indistinguishable from levels detected in biological material free blanks. NOS activity was unchanged in cytosolic aliquots maintained at all other temperatures.

Effects of protease inhibitors

Previous publications detailing NOS investigations have used protease inhibitors in enzyme preparations. To determine if this was necessary when working with *A. pallida* preparations, mixtures of protease inhibitors were administered to cytosolic samples and enzyme activity determined and compared with untreated preparations. Chymostatin (a serine and cysteine protease inhibitor that inhibits chymotrypsin, papain and cathepsins A, B, and C), leupeptin (an inhibitor of serine and cysteine proteases), pepstatin (inhibitor of acid proteases, including pepsin, renin and cathepsin) and trypsin inhibitor were used. Protease inhibitor mixtures were added to cytosol just prior to incubation initiation by cofactor mix addition. Final concentrations of 10 µg/ ml or 50 µg/ ml of each inhibitor were added. Control cytosolic aliquots from the same preparation were incubated with cofactor mix alone.

Results

Addition of 10 or 50 $\mu\text{g/l}$ of chymostatin, leupeptin and pepstatin with or without trypsin inhibitor had no effect on detected levels of NOS activity.

Determination of baseline levels of NOS activity

Inter individual variance

Multiple individual anemones were assayed to compare baseline levels of enzyme activity.

In each case anemones from a single stock maintained in identical conditions were used.

Results

Levels of enzyme activity varied between individuals and between assays, (table 3.2). Rates of citrulline formation determined in individual anemones ranged from 0.47 ± 0.00 to 9.96 ± 0.06 $\text{pmole } \mu\text{g protein}^{-1} \text{ min}^{-1}$. The highest rates of formation were detected in recently collected anemones (mean 6.71 ± 1.85 $\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$, $n=10$, assay performed within 10 days of collection). Anemones maintained for long periods of time under laboratory conditions had rates of citrulline formation in the range $0.47\text{-}3.74$ $\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$.

Cultured symbiotic and aposymbiotic *A. pallida*

Previous work has identified a marked difference in NOS levels in cultured zooxanthellae compared to those freshly isolated from symbiosis (Trapido-Rosenthal *et al.*, 1996). The significance of the symbiotic association with zooxanthellae in terms of host tissue levels of NOS were investigated by analysis of cytosol from aposymbiotic (essentially zooxanthellae free anemones) and standard symbiotic anemones. Aposymbionts were prepared as detailed in section 2.1.

Results

Similar levels of NOS activity were detected in cytosolic preparations from aposymbiotic and symbiotic individuals (table 3.3).

Anemone collection/maintenance details	Mean pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation \pm s.d.	Range (pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation)	n
Long term cultured clones	0.75 ± 0.18	0.55-0.95	3
Recently collected (three days prior to assay, 15.05.98)	0.8 ± 0.26	0.47-1.16	7
Recently collected (10 days prior to assay, 28.09.99)	6.71 ± 1.85	4.05-9.96	10
Recently collected (14 days prior to assay, 28.09.99)	3.64 ± 0.97	2.67-5.28	6
Recently collected (one day prior to assay, 28.12.99)	0.76 ± 0.15	0.65-1.00	3
Recently collected (13 days prior to assay, 10.12.99)	1.40 ± 0.64	0.82-1.97	3

Table 3.2. Rates of citrulline formation in *A. pallida* with different collection histories. Individual anemones were assayed in triplicate; mean and s.d. values were calculated from the combined results from all individuals in each experimental group. The range shown is the minimum and maximum rate of formation shown by anemones assayed. N indicates the number of individual anemones assayed in each group (each assayed in triplicate). Anemones were maintained in standard conditions until just prior to assay preparation.

Anemone type	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ \pm s.d.
Symbiotic	1.6 ± 0.36
Aposymbiotic	1.9 ± 0.23

Table 3.3. Rates of citrulline formation in cultured symbiotic and aposymbiotic (essentially zooxanthellae free) *A. pallida* (n=2). Values presented are means \pm s.d. of triplicate determinations.

Citrulline formation in zooxanthellae

NOS activity has been observed in zooxanthellae isolated from *A. pallida* (Trapido-Rosenthal *et al.*, 1996) but basal rates of activity have not previously been defined. We investigated the rates of enzyme activity in zooxanthellae freshly isolated from *A. pallida* (prepared as detailed in section 2.1). The citrulline assay was run with intact zooxanthellae cells or cytosol derived from disrupted cells. Zooxanthellae density counts (section 2.8) were performed on pre- and post sonicated preparations to allow calculation of the number of disrupted cells. Zooxanthellae NOS values were expressed in terms pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$ and pmoles $\text{cell}^{-1} \text{ min}^{-1}$.

Results

Citrulline formation in intact zooxanthellae cells was indistinguishable from biological material free blanks. Citrulline formation was detected in zooxanthellae cytosol (table 3.4).

	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ $\pm \text{s.d.}$	Pmoles citrulline zooxanthellae cell min^{-1} $\pm \text{s.d.}$
Sample 1	0.16 ± 0.00	$6.04 \times 10^{-7} \pm 6.84 \times 10^{-9}$
Sample 2	0.11 ± 0.09	$4.52 \times 10^{-7} \pm 4.52 \times 10^{-8}$

Table 3.4. Rates of citrulline formation in zooxanthellae cytosolic preparations. Values given are means of triplicate determinations $\pm \text{s.d.}$ Zooxanthellae from samples 1 and 2 were from independent anemones.

Coral Species

A pilot study was conducted to investigate the presence and rates of basal citrulline formation were investigated in the scleractinian coral species *Montastrea franksi*, *Montastrea cavernosa*, *Agaricia* sp., *Madracis mirabilis* and *Madracis decactis*. In order to minimise damage to the coral reef collection sites, only one or two samples of each species were collected. Collection and preparation methods are detailed in chapter two.

Results

NOS activity was detected by the citrulline assay in all coral species investigated (table 3.5).

Rates of citrulline formation ranged from 0.18-1.53 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation.

Coral species	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ incubation \pm s.d.	Collection site	n
<i>Madracis decactis</i>	0.18 ± 0.01	Shelly Bay Shoals	2
<i>Madracis mirabilis</i>	0.24 ± 0.06	Shelly Bay Shoals	2
<i>Montastrea cavernosa</i>	1.53 ± 0.07	Hog Breaker	1
<i>Montastrea franksi</i>	1.31 ± 0.25	Hog Breaker	2
<i>Agaricia</i> sp.	0.44 ± 0.08	Halls Island	1

Table 3.5. Rates of citrulline formation in a selection of scleractinian coral species from Bermuda. Values shown are means of triplicate determinations \pm s.d., n= number of samples from separate colonies each run in triplicate.

NOS enzyme kinetics

Substrate kinetics

Investigations were carried out to investigate rates of enzyme activity over a range of substrate concentrations. As the kinetic parameters were determined with use of an unpurified form of the NOS enzyme, it is appropriate to refer to the determined values as the apparent V_{max} (maximum rate of reaction) and apparent K_m (concentration of substrate when the velocity is half-maximal). Aliquots of anemone cytosol from a single preparation were incubated with exogenous arginine at concentrations between 0 and 300 μM (maintaining a constant ratio of labelled to unlabelled arginine). Rates of citrulline formation were plotted against substrate concentration and the kinetics investigated using appropriate mathematical models (detailed in section 2.9).

Results

Rates of citrulline formation as a function of arginine concentration are shown in figure 3.1. At L-arginine concentrations between 200 and 300 μM rates of NOS activity were stable (11.39 ± 1.00). Non-linear regression of this data is shown in figure 3.2. This transformation gave estimates of K_m equal to 132.9 μM L-arginine (apparent K_m) and V_{max} equal to 17.7 $\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$ (apparent V_{max}).

Time course of activity

The time period for which linearity in rates of formation is maintained in assays is dependent on the type of NOS being studied and the conditions of the assay (Knowles and Moncada, 1994). Experiments were conducted with variable incubation times to determine if the rate of citrulline formation time was linear at 30 minutes incubation time. Rates of citrulline formation were determined in incubations terminated 5-120 minutes after initiation.

Results

Rates of citrulline formation ($\text{pmoles citrulline } \mu\text{g protein}$) were linear up to 10 minutes incubation time ($y=3.89x$, R^2 0.99, intercept 0). At incubation times between 10-40 minutes, overall formation of citrulline increased (figure 3.3), but at a decreasing rate. Beyond 40 minutes, the rate of formation of citrulline declined.

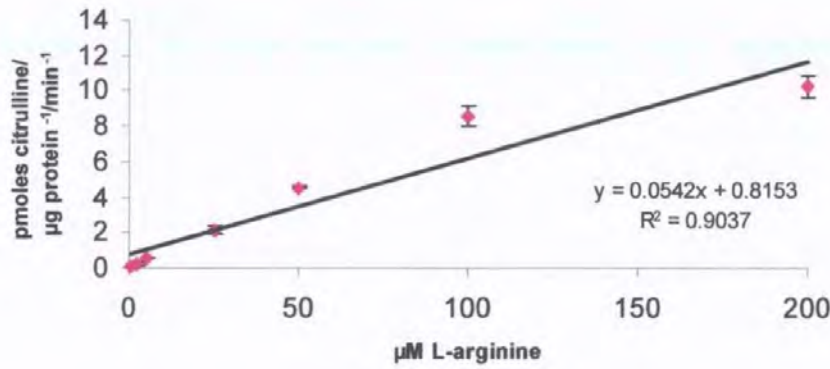


Figure 3.1. Rates of citrulline formation in *A. pallida* cytosol incubated with exogenous substrate concentrations between 0 and 200 μM . Incubations were 30 minutes at 25°C. Multiple aliquots of a single cytosolic preparation were used. Values shown are means of triplicate determination. Error bars show standard deviations.

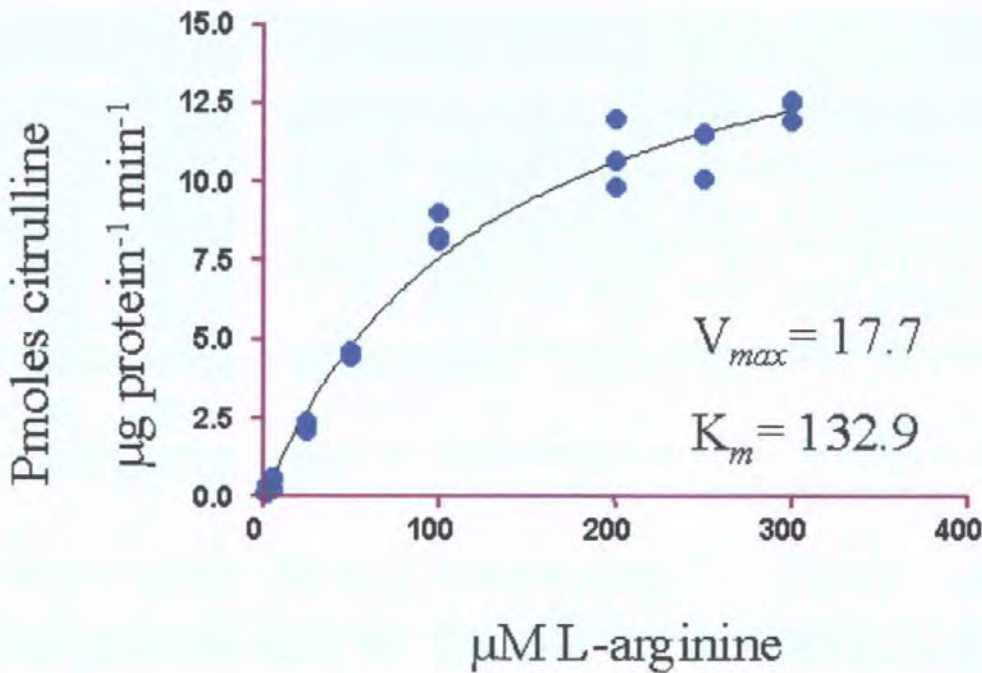


Figure 3.2. Kinetics of *A. pallida* NOS activity. Production of ^3H -citrulline was measured as a function of ^3H -arginine concentration. The Lineweaver-Burke transformation of the resulting data is displayed, along with the resulting estimates of K_m and V_{max} .

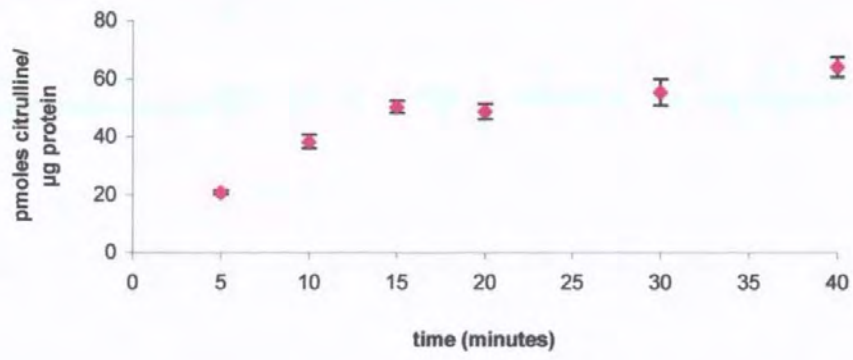


Figure 3.3. Rates of citrulline formation in *A. pallida* cytosol incubated for 5-40 minutes. Incubations were initiated by addition of cofactor mix. Values shown are means of triplicate determinations. Error bars show standard deviations.

Activity in relation to biological material

Volumes of biological material between 2-120 μl (containing 1.1- 110.7 μg protein) were incubated to investigate the linearity of enzyme activity response. Addition of arginine mix initiated incubations.

Results

Enzyme activity increased linearly ($R^2=1$, $y=1.5472x+0.4806$) relative to protein content up to 11.1 μg protein where the rate of citrulline formation was 17.6 ± 0.7 pmoles min (figure 3.4). Rates of citrulline formation were at a maximum when 27.7 μg protein were added (23.2 ± 3.2 pmoles min); addition of samples with higher protein contents did not further increase rates of formation.

Effects of exogenous L-arginine on levels of *A. pallida* NOS activity

The effect of a high concentration of unlabelled substrate on NOS activity was examined by comparing citrulline formation from 20 μM ^3H -L-arginine in the presence and absence of 1 mM unlabelled L-arginine. NOS activity was also compared in aliquots of a single preparation incubated with or without addition of 20 μM unlabelled arginine (concentration equal to that in standard incubation mix).

Results

Addition of 1 mM unlabelled L-arginine to cytosolic preparations reduced citrulline formation by 81.8% (figure 3.5) compared to the control condition (0.37 ± 0.03 and 2.03 ± 0.06 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ respectively). Similar rates of citrulline formation were detected in samples assayed in the presence or absence of 20 μM unlabelled exogenous arginine (0.34 ± 0.6 and 0.35 ± 0.05 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ respectively).

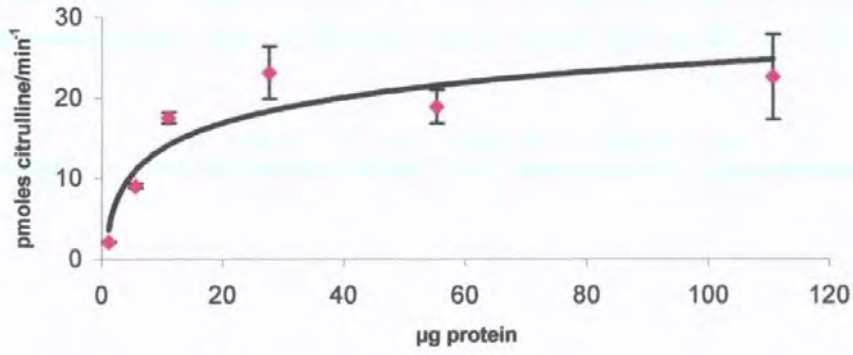


Figure 3.4. Rates of citrulline formation in relation to *A. pallida* cytosolic protein content. Multiple aliquots from a single cytosolic preparation were used. Values shown are means of triplicate determinations. Error bars represent standard deviations.

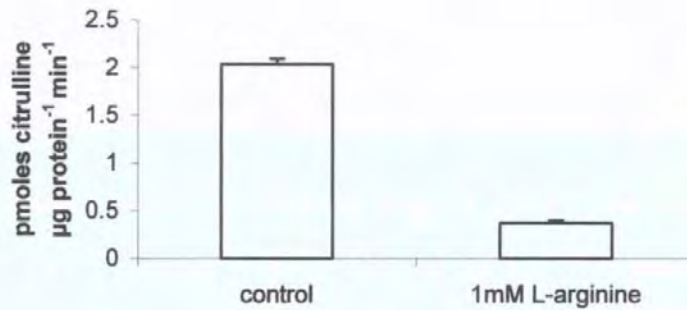


Figure 3.5. Rates of citrulline formation in *A. pallida* cytosolic preparation incubated with standard (20 µM, control) or an excess (1 mM) of exogenous arginine. Cytosolic aliquots were from a single preparation. Values shown are means of triplicate determinations. Error bars represent standard deviations.

Exogenous cofactor effects on levels of *A. pallida* NOS activity

Due to the unpurified nature of the tissue homogenate used for enzyme characterisation it was important to evaluate the effect of cofactor addition or exclusion from the enzyme assay mix. In addition, it was of interest to determine cofactor requirements for *A. pallida* NOS for comparison with those previously defined for other isoforms. Several approaches using multiple aliquots from single cytosolic preparations of *A. pallida* were made to investigate the influence of exogenous cofactors on NOS activity. Enzyme activity was investigated in preparations incubated with or without cofactor additions. In order to determine the influence of calcium on enzyme activity, the effect of addition of the calcium chelator EGTA (5 mM) was investigated. To remove endogenous cofactors, cytosolic samples were pre-run through PD-10 Sephadex G25 M columns (Supelco USA) equilibrated with HB as previously documented by Radomski *et al.*, (1991). Sample volumes of 2.5 ml were added to the column and eluted by addition of 2 ml HB. After allowing 2 ml of flow through, a 2 ml aliquot of eluant was collected and used in incubations. Citrulline formation was compared in aliquots subject to treatments outlined in table 3.6.

Results

With and without cofactor addition

Slightly higher rates of citrulline formation were observed in cytosolic aliquots of *Aiptasia pallida* and the coral species *Madracis mirabilis* and *Madracis decactis* when arginine mix was added relative to samples to which full cofactor mix was added (table 3.7).

Control	Treatment
Arginine mix	Cofactor mix
Arginine mix	Arginine mix + single cofactor addition
Cofactor mix	Cofactor mix with single cofactors excluded
Cofactor mix	Cofactor mix + 5 mM EGTA
Cofactor mix with CaCl ₂ excluded	Cofactor mix with CaCl ₂ excluded + 5 mM EGTA
Arginine mix	Arginine mix + 5 mM EGTA
Standard cytosol + arginine mix	Cytosol with endogenous cofactors removed + arginine mix
Cytosol with endogenous cofactors removed + arginine mix	Cytosol with endogenous cofactors removed + NADPH, CaCl ₂ or calmodulin + arginine mix

Table 3.6. Outline of incubations used to investigate cofactor requirements of *A. pallida* NOS. Except where otherwise stated, standard cytosolic aliquots were used. Single cofactors were added in concentrations equivalent to those in standard cofactor mix (final concentrations: 1 mM NADPH, 1.25 mM CaCl₂, 1 mM DTT, 10 μ M BH₄, 150 units calmodulin). Arginine mix and cofactor mix were added in quantities to give final concentrations of 20 μ M L-arginine.

Species	With cofactors pmoles citrulline μ g protein ⁻¹ min ⁻¹ incubation \pm s.d.	Without cofactors pmoles citrulline μ g protein ⁻¹ min ⁻¹ incubation \pm s.d.
<i>A. pallida</i>	2.10 ± 0.13	2.38 ± 0.13
<i>Madracis mirabilis</i>	0.24 ± 0.06	0.37 ± 0.10
<i>Madracis decactis</i>	0.18 ± 0.01	0.32 ± 0.10

Table 3.7. Rate of citrulline formation in cnidarian cytosol incubated with and without exogenous cofactor addition. Incubations were initiated by addition of exogenous arginine. Values given are means of triplicate determinations \pm s.d.. Results shown for *A. pallida* are from two separate experiments using pooled tissue from five anemones in each assay. Two colonies of each coral species were assayed.

EGTA

No consistent effect of cytosolic extract treated with EGTA was observed. Incubation with EGTA and full cofactor addition lead to small variations in the rate of citrulline formation relative to untreated controls. Addition of EGTA with or without CaCl_2 had a negligible effect on activity of NOS.

Single cofactor inclusion

Addition of NADPH resulted in statistically significant increases in rates of citrulline formation (table 3.8). Rates of citrulline formation were elevated in samples to which biopterin or calmodulin were added relative to controls but these were not statistically significant. No consistent effect was evident from addition of CaCl_2 .

Cofactor included	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1} \pm \text{s.d.}$	% change from control	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1} \pm \text{s.d.}$	% change from control
Control (substrate only)	0.39 ± 0.01	-	0.52 ± 0.02	-
NADPH	0.48* ± 0.01	+23.1	0.59* ± 0.3	+13.5
CaCl_2	0.32 ± 0.05	-17.9	0.54 ± 0.04	+3.8
DTT	0.44 ± 0.07	+12.8	0.57 ± 0.05	+9.6
Biopterin	0.40 ± 0.02	+2.6	0.47 ± 0.01	-9.6
Calmodulin	0.43 ± 0.02	+10.26	0.56* ± 0.02	+7.7
Standard cofactor mix	0.28* ± 0.02	-28.2	0.55 ± 0.11	+5.8

Table 3.8. Rates of citrulline formation in cytosolic preparations treated with single cofactors. Values given are means of triplicate determinations \pm s.d.. Results of two independent experiments are shown. Cofactors were added to give concentrations equivalent to those resulting from addition of standard cofactor mix. Asterisks mark values statistically significantly different ($p < 0.05$) from controls.

Single cofactor exclusion

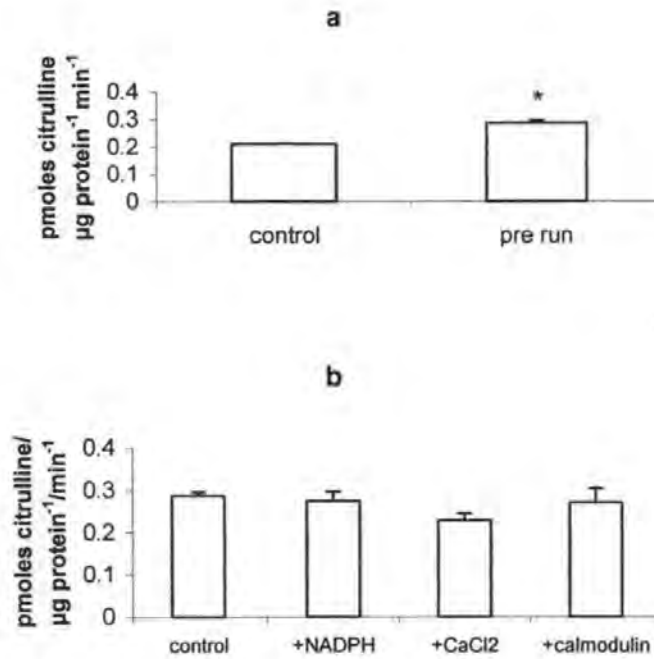
Exclusion of single cofactors from the standard cofactor mix caused slight variations in the rates of NOS enzyme activity (table 3.9). No consistent effect of exclusion of NADPH, CaCl_2 , or calmodulin were observed.

Cofactor excluded	Pmoles citrulline μg protein ⁻¹ min ⁻¹ \pm s.d.	% change from control	Pmoles citrulline μg protein ⁻¹ min ⁻¹ \pm s.d.	% change from control
Control (standard cofactor mix)	0.35 ± 0.06	-	0.55 ± 0.01	-
NADPH	0.34 ± 0	-2.9	0.61 ± 0.08	+10.9*
CaCl_2	0.34 ± 0.01	-2.9	0.65 ± 0	+18.2
DTT	0.37 ± 0.01	+5.7	-	-
Biopterin	0.33 ± 0.02	-5.7	-	-
Calmodulin	0.32 ± 0.03	-8.6	0.80 ± 0.26	+45.5*

Table 3.9. Rates of citrulline formation in cytosolic preparations from which single cofactor were excluded. Values given are means of triplicate determinations \pm s.d. Results of two independent experiments are shown. Cofactors were added to give concentrations equivalent to those resulting from addition of standard cofactor mix. Asterisks mark values statistically significantly different ($p < 0.05$) from controls.

Low molecular weight component exclusion

Cytosol eluted through a G25 column treated with arginine mix alone had 38% higher level of citrulline formation than non-eluted cytosol (figure 3.6a). Eluted cytosol incubated with NADPH, or calmodulin showed rates of citrulline formation that were little changed from eluant treated with arginine mix alone (figure 3.6b). Although not statistically significant ($p > 0.05$), incubation of cytosol in 1.25 M CaCl_2 resulted in a 20.7% reduction in citrulline formation relative to eluant treated with arginine mix alone (figure 3.6 b).



Figures 3.6a and 3.6b. Rates of citrulline formation in *A. pallida* cytosol. Values shown are means of triplicate determinations. Error bars show standard deviations. 3.6a depicts rates of formation in standard cytosol (control) and cytosol eluted through a G25 column (pre run) both incubated with arginine mix alone. The asterisk indicates a value statistically significant different from that of the control ($p < 0.05$). 3.6b depicts rates of citrulline formation in G25 eluted cytosol incubated with arginine mix alone (control) or co-incubated with the single cofactors NADPH (1 mM), CaCl_2 (1.25 mM) or calmodulin (150 units) (values in parentheses are final concentrations).

Effects of arginine analogues on levels of *A. pallida* NOS activity

The effect of treatment of *A. pallida* with a number of arginine analogues previously reported to be NOS inhibitors was investigated. Among the arginine analogues identified and receiving significant use are N^W-nitro-L- arginine (L-NNA), N^W-nitro-L- arginine methyl ester (L-NAME), N^W-amino-L- arginine (L-NAA), N^W-Methyl-L-arginine-acetate (L-NMA) and N^δ- iminoethyl-L- ornithine (L-NIO) (Griffith and Gross, 1996). L-NMA has been described as the prototypic NOS inhibitor (Griffith and Gross, 1996) and is the most widely used NOS inhibitor (Franchini *et al.*, 1995b).

Arginine analogues were administered to cytosolic preparations of *A. pallida*. Arginine analogue solutions were made up in HB. The arginine analogues L-NAME, L-NMA, D-NMA and L-NNA were incubated with cytosolic preparations and the subsequent rates of citrulline formation determined. Investigation of the response of NOS to concentrations of L-NMA between 0.1 and 1500 μ M enabled calculation of an inhibition constant (K_i) enabling comparison with previously reported inhibition characteristics. Cytosolic preparations were either pre-incubated or incubated during the standard citrulline assay (section 2.2). In a separate experiment, cytosolic aliquots from a single preparation were pre-run through a Dowex ion exchange column to remove endogenous L-arginine prior to inhibitor treatment.

Results

Cytosol incubation

No statistically significant difference was detected between control incubations and preparations incubated with 1 or 3 mM L-NAME or L-NNA. Cytosolic preparations, with either intact or removed endogenous arginine, incubated with 5 mM L-NNA had NOS activity levels 15% lower than control values (figures 3.7a and b).

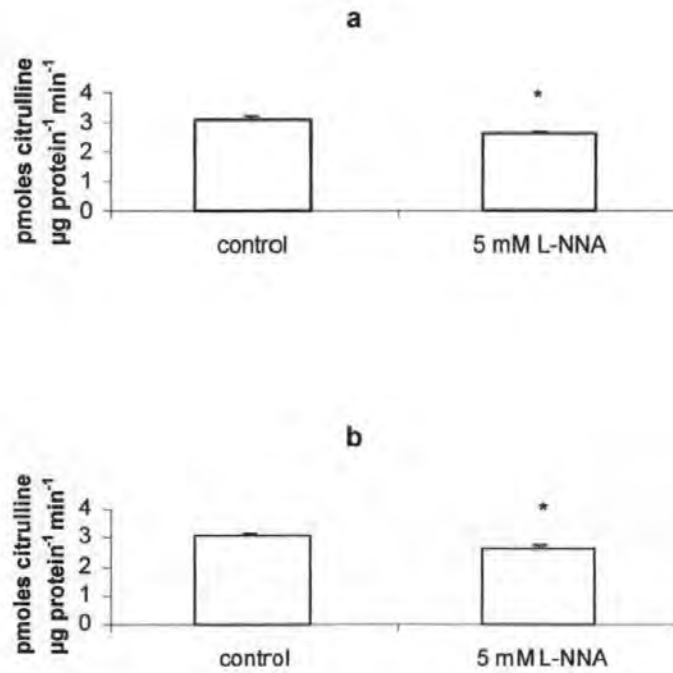


Figure 3.7a and 3.7b. Rates of citrulline formation in *A. pallida* cytosolic preparations incubated with the arginine analogue L-NNA. 3.7a shows G25 eluted cytosol, 3.7b shows standard cytosol (non eluted). Controls were incubated without L-NNA. Values shown are means of triplicate determinations. Error bars show standard deviations. Asterisks mark values statistically significant different from controls.

Ten-minute pre-incubation of cytosolic preparations prior to standard assay with 1 mM L-NMA or D-NMA resulted in statistically significant reductions in citrulline formation relative to controls (figure 3.8); the L- enantiomer caused a little more than double the inhibition of the D- form (74% and 36% activity reductions respectively). Cytosolic samples incubated with L-NMA in the range ≤ 0.1 mM L-NMA (figure 3.9), had rates of citrulline formation that were not significantly different from untreated controls. Cytosol treated with concentrations 0.5-1.5 mM L-NMA had statistically significantly reduced rates of citrulline formation ($p < 0.05$) relative to controls (figure 3.9). Rates of NOS activity were just 11.2% of control levels in samples incubated with 1.5 mM L-NMA. An apparent (so called as a result of the unpurified nature of the biological preparation used) K_i of 1014 μM was determined for L-NMA using the graph pad method of Cheng and Prusoff (1973). Concentration of radioligand used in the calculation was 20 μM along with the previously calculated estimate of $K_m = 132.9$.

Effects of an exogenous NO donor on levels of *A. pallida* NOS activity

Investigations of the response of *A. pallida* NOS to exogenous NO were conducted by administration of the NO donor sodium nitroprusside (SNP) to whole or cytosolic preparations of *A. pallida*. SNP is an inorganic complex in which the NO is formally bound as NO^+ (Feelisch and Stamler, 1997a). SNP has been widely used as a NO donor; in biological systems both enzymatic and non-enzymatic NO release from SNP may occur (Feelisch and Stamler, 1997a).

Whole anemones were incubated in solutions of 20 or 100 μM sodium nitroprusside (SNP) for 24 hours prior to assay, or cytosol was incubated with 2 mM SNP for 30 minutes during standard assay incubation. Levels of NOS activity were determined by standard assays

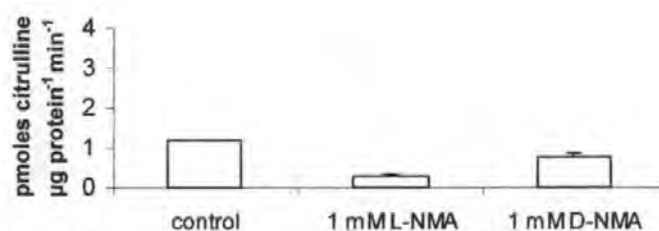


Figure 3.8. Rates of citrulline formation in *A. pallida* cytosolic preparations incubated with L-NMA or D-NMA. Control cytosol was incubated without addition of NMA. Cytosolic aliquots were from a single preparation. Values shown are means of triplicate determinations. Error bars show standard deviations.

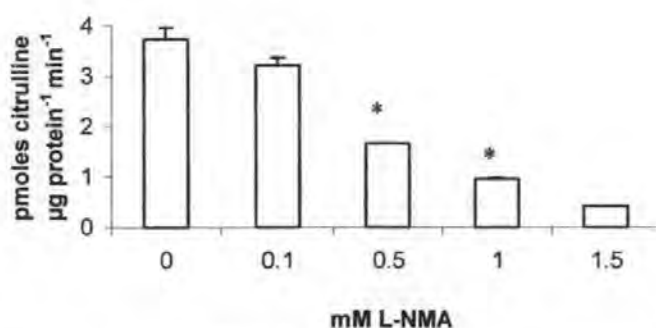


Figure 3.9. Rates of citrulline formation in *A. pallida* cytosolic preparations incubated with L-NMA between 0.1-1.5 mM. Control cytosol was incubated without addition of L-NMA. Cytosolic aliquots were from a single preparation. Values shown are means of triplicate determinations. Error bars show standard deviations. Asterisks mark values statistically significant different from controls.

initiated by addition of cofactor mix. Physiological responses of whole anemones incubated in SNP solutions are described in chapter 5.

Results

No statistically significant difference in NOS activity was detected in anemones incubated for 24 hours in 20 μ M SNP, but activity was statistically significantly reduced by incubation in 100 μ M for the same period relative to control (table 3.10). Cytosol incubated with 2 mM SNP during the 30-minute citrulline assay incubation had statistically significantly lower levels of NOS activity than untreated controls (table 3.10).

Treatment	Control (pmoles citrulline μ g protein ⁻¹ min ⁻¹ \pm s.d.)	SNP treatment (pmoles citrulline μ g protein ⁻¹ min ⁻¹ \pm s.d.)	% change
a Whole anemone incubation in 20 μ M SNP	0.78 \pm 0.03	0.85 \pm 0.02	+8.97
b Whole anemone incubation in 100 μ M SNP	0.78 \pm 0.03	0.58 \pm 0.01*	-25.64
c Cytosol incubated with 2 mM SNP	0.78 \pm 0.03	0.49 \pm 0.01*	-37.18

Table 3.10. Rates of citrulline formation in: rows a and b, cytosolic preparations of whole anemones pre-incubated in SNP solution for 24 hours prior to assay, control values were obtained from anemones maintained in LNSW (n=6 individuals per treatment) and row c, cytosolic preparations incubated with SNP during standard assay incubation (30 minutes), control and treated cytosol samples were aliquots taken from a single anemone preparation (prepared from pooled homogenate from six individuals). Values given are means of triplicate determinations \pm s.d.. Asterisks denote values that are statistically significantly different from controls ($p < 0.05$).

Chapter Three

Effects of lipopolysaccharide treatment on levels of *A. pallida* NOS activity

Lipopolysaccharide (LPS) is a bacterial polysaccharide that is a proven immunostimulant in many organisms. Previous work has shown LPS to be a potent elicitor of a respiratory burst in amoebocytes from the sea anemone *Actinia equina* (Smith and Hutton, 1995). The ability of LPS to induce NOS enzymes has been documented in other invertebrate species (i.e. molluscan haemocytes, Conte and Ottaviani, 1995 and crustacean haemocytes, Radomski *et al.*, 1991). Experiments were designed to investigate the effects of pre incubation of whole anemones with LPS on NOS activity in cytosolic preparations. Whole anemones were injected into the pharynx via the mouth with a flat tipped Hamilton syringe with 20 µg LPS then incubated for 24 hours. In separate experiments, whole anemones were incubated in 20 or 40 µg/ml LPS solution 24 hours prior to assay. In each case, LPS was made up in LNSW. Assay incubations were initiated by addition of cofactor mix.

Results

The effects of LPS exposure of whole anemones on rates of citrulline formation were variable and irreproducible (table 3.11).

Treatment	Control (pmoles citrulline µg protein ⁻¹ min ⁻¹ ± s.d.)	LPS treatment (pmoles citrulline µg protein ⁻¹ min ⁻¹ ± s.d.)	% change
20 µg LPS injection	0.39 ±0.01	0.48 ±0.05	+23.1
20 µg LPS injection	0.46 ±0.02	0.46 ±0.01	0
20 µg/ml LPS incubation	0.78 ±0.03	1.17 ±0.01	+50.0
20 µg/ml LPS incubation	0.46 ±0.02	0.28 ±0.01	-39.0
40 µg/ml LPS incubation	0.78 ±0.03	1.01 ±0.02	+29.5

Table 3.11. Rates of citrulline formation in anemones treated with LPS. Values given are means of triplicate determinations ± s.d.. Cytosol was prepared from multiple anemones (5-10) for controls and treatments. Anemones were injected with LPS and assayed 24 hours later, or they were incubated for 24 hours in solutions of LPS. Data shown from identical LPS treatments are from separate experiments.

3.2 Discussion

The accuracy and hence the value of the results presented in this chapter determined from the citrulline assay are undermined by the lack of careful preliminary work to determine optimal assay conditions. Specific problems are identified and discussed in subsequent text, along with suggestions for eliminating these problems in future studies. In spite of the problems with the results, a significant amount of preliminary data is reported that can be used, albeit with caution, both for comparison with other research findings and as a foundation for future studies.

The citrulline assay

Measurement of the formation of citrulline was used as an indirect approach to monitoring the activity of NOS. As NOS enzymes are responsible for catalysing generation of NO and/or redox species of NO, differences in NOS activity level indicated differences in the rate of production of these products.

Intra assay variance

The citrulline assay utilised in this research was shown to be reproducible in determining rates of citrulline formation in cytosolic preparations of *A. pallida*. As a result, confidence was gained that any differences detected in rates of citrulline formation in aliquots of cytosol (from a single preparation) subject to different treatments were responses to applied treatments rather than the result of intra assay variance.

Contrary to opinion of Johansson and Carlberg (1995) that the citrulline assay is not capable of detecting small amounts of NOS activity in invertebrate tissues, the assay was found to be suitably sensitive for the detection of NOS activity in cytosolic prepared from homogenates of whole body tissues of *A. pallida*. Our findings are thus in agreement with others who have used the citrulline assay to determine NOS activity in various invertebrate

preparations (Colasanti *et al.*, 1997; Salleo *et al.*, 1996; Radomski *et al.*, 1991; Conte and Ottaviani, 1995; Moroz *et al.*, 1996; Colasanti *et al.*, 1995). The citrulline assay has been reported to be sensitive enough to detect the formation of <100 nM product (Knowles and Moncada, 1994). However, when the citrulline assay is used to determine NOS activities in relatively crude preparations, a variety of factors have potential to increase inter assay variance. These factors relate to assay-to-assay variations in the levels of endogenous substrates, inhibitors and cofactors.

Since completion of the research documented in this thesis it has been recognized that in the initial stages of this thesis research, it would have been informative to run the citrulline assay with a NOS preparation with a known level of enzyme activity (as available from scientific product manufacturers). This would have enabled determination of the accuracy of the assay in predicting enzyme activity. In addition, by spiking standard preparations of *A. pallida* with a NOS preparation with a known amount of enzyme activity, it may have been possible to gauge the extent to which endogenous components in *A. pallida* preparations were interfering with the citrulline assays ability to predict enzyme activity levels. These results could then have been applied to all subsequently generated data.

Endogenous L-arginine

The lack of difference in rates of enzyme activity in cytosolic aliquots incubated with or without intact endogenous L-arginine observed in this study indicate that the presence of endogenous L-arginine does not influence the observed rates of citrulline formation. By providing exogenous L-arginine to citrulline assay incubations (in the form of either arginine mix or standard cofactor mix), the effects of endogenous arginine are likely to be diluted to the extent that their influence on rates of enzyme activity are insignificant.

L-valine and L-ornithine

The possibility that formation of ^3H -citrulline was occurring through the activity of arginase and ornithine transcarbamylase (which can indirectly synthesise L-citrulline from L-arginine) (Blachier *et al.*, 1991) was ruled out as rates of citrulline formation were unchanged in samples incubated with the arginase inhibitors L-valine (Knowles and Moncada, 1994) and L-ornithine, (Giraldez and Zweier, 1998) compared to untreated samples. Increased citrulline formation has been noted in rat alveolar macrophages treated with an arginase inhibitor (Blachier *et al.*, 1991), presumably due to increased L-arginine availability (Giraldez and Zweier, 1998). The findings of the research reported here show no increase in citrulline formation in samples co-incubated with L-ornithine or L-valine, providing evidence that arginase activity is not interfering with determination of NOS activity in cytosolic preparations of *A. pallida*. These findings are of crucial importance when working with whole body preparations in which multiple inter-related enzymatic pathways may be present. The presence of additional NOS-independent routes of L-citrulline production were not reported at the time of this study and therefore were not investigated in this study. Further studies should ensure that alternative routes of L-citrulline production are not interfering with accurate determinations of NOS activity levels.

Enzyme stability

NOS activity levels were shown to be unchanged in cytosolic preparations maintained over a two-hour period between -70 and 0°C indicating that the enzyme was stable beyond the requirements of the assay technique. Results showing no change in NOS activity in samples incubated with protease inhibitors indicate that proteolytic enzymes added directly to cytosolic preparations have an insignificant effect on NOS enzyme activity in cytosolic preparations of *A. pallida* within the time frame of the citrulline assay. Protease inhibitors were consequently not added to biological preparations. Subsequent to completing these

experiments, it has been recognized that protease inhibitors are typically added to homogenisation buffers rather than directly to cytosol. Future studies should investigate the effect of protease inhibitors added to homogenisation buffer on NOS activity. This is of particular importance when using a hypotonic buffer like HB. Application of HB to tissue preparations is likely to result in bursting of organelles (due to osmotic shock) including lysosomes with subsequent liberation of lysosomal enzymes. The effect of this on NOS activity was not documented in this research but should be investigated in future studies. Specifically, the usefulness of: protease inhibitors, standardized time periods between homogenisation and assay and, addition of sucrose or salt to HB should be evaluated.

Basal levels of NOS activity

The results presented here indicate that the sea anemone *A. pallida* (both symbiotic and aposymbiotic forms), *Symbiodinium* spp. (the symbiotic zooxanthellae associated with *A. pallida*), and a variety of scleractinian coral species possess measurable NOS activity.

Basal levels of NOS activity in zooxanthellae freshly isolated from *A. pallida*

Disrupted zooxanthellae freshly isolated from *A. pallida* were shown to have detectable levels of NOS activity. These findings are in agreement with those of Trapido-Rosenthal *et al.*, (1996). To the authors' knowledge, determination of basal levels of NOS activity in zooxanthellae is unprecedented. Investigations into the significance of zooxanthellae NOS activity and subsequent NO production in the symbiotic association with a cnidarian host are recommended. Of particular interest is the potential role played by NO as a mediator of communication between host and zooxanthellae. In addition to reporting the presence of NOS in zooxanthellae, the study of Trapido-Rosenthal *et al.*, (1996) provides results that show that zooxanthellae NOS activity is linked to symbiotic status suggesting a role for NOS within the symbiotic assemblage of *A. pallida* and *Symbiodinium* spp..

Basal levels of NOS activity in aposymbiotic and symbiotic anemones

This preliminary study indicated no difference in levels of NOS activity in aposymbiotic and symbiotic animal tissue preparations from long term cultured anemones but further work is recommended using larger sample sizes and replicate experiments to validate these findings. As the biochemistry of the host is affected by activities of both host and zooxanthellae, it would be of interest to investigate the influence of a range of parameters on host levels of enzyme activity in both symbiotic and aposymbiotic anemones.

Basal levels of NOS activity in a range of scleractinian corals

Preliminary investigations with cytosolic preparations of scleractinian coral tissues showed detectable levels of enzyme activity in all species tested. Levels of enzyme activities detected in corals were below, or within the range of values detected in *A. pallida* preparations. These data are the first to show and define basal rates of NOS activity in scleractinian coral species. As such they extend knowledge of NOS levels in cnidarians and invertebrates in general. Further work is needed to verify these preliminary results with use of larger sample numbers and a citrulline assay optimised for use with these preparations.

Basal levels of NOS activity in *A. pallida*

The range of levels of NOS activity determined in *A. pallida*, at between 0.47 ± 0 to 9.96 ± 0.06 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$ include values substantially higher than those previously reported for a variety of invertebrate preparations. NOS activity has been reported to be equal to 0.51 ± 0.02 pmoles $\text{mg}^{-1} \text{ min}^{-1}$ (approximately 0.03 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$) in cnidarian acontial preparations (Salleo *et al.*, 1996), and approximately 0.10 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$ in molluscan haemocytes (Conte and Ottaviani, 1995). Use of different species, different types of biological preparations and assay methods and use of a non-

optimised assay, prevents direct comparison of results. Use of BSA as standard in the Bradford assay in determination of protein contents of cnidarian tissues has been reported to result in consistent underestimates of protein values owing to different extinction coefficients of cnidarian protein versus BSA (Zamer *et al.*, 1989). Underestimation of protein content of *A. pallida* cytosolic preparations would result in the overestimation of protein normalised rates of enzyme activity. This may account for the higher reported rates of enzyme activity in *A. pallida* relative to reports from other species and tissues. In addition, possible underestimation of protein contents of the tissues of *A. pallida* has implications for the kinetic parameter V_{max} defined by this study. Further studies are highly recommended to investigate the possibility and degree of underestimation of protein content in *A. pallida* preparations. Use of isolated protein from sea anemones as protein standard, as suggested by Zamer *et al.*, (1989), as a means of increasing the accuracy of protein determination may prove useful in future studies.

Considerable variations in levels of citrulline formation in cytosolic preparations of *A. pallida* were observed over the experimental period. Possible sources of variance in determined levels of activity are numerous and include biotic, abiotic, physiological and biochemical factors. At least some of the observed variations in NOS activity may be attributed to the method of preparation of biological material. Variation of tissue dilution of preparations may have resulted from differences in: anemone biomass, degree of tissue hydration and water content of the pharynx. These factors are likely to have influenced the determined levels of NOS activity. Anemones of similar sizes were selected for use in experiments but, owing to differential states of contraction and expansion, the biomass of selected individuals was at times quite variable. Tissue dilution variations have implications for final concentrations and ratios of endogenous: exogenous substrate, final cofactor concentrations and protein contents of cytosolic preparations. The influence of tissue dilution on detected rates of NOS activity must be eliminated in future studies by

standardisation of the amount of protein added to assay incubations. This could be achieved by determining protein levels in cytosolic preparations prior to running citrulline assays and adjusting cytosol volumes used or by standardizing the ratio of biological material: homogenisation buffer used. Alternatively, the average wet: dry weight ratios of *A. pallida* could be determined using a large number of anemones (minimum 100) and these result applied to experimental anemones weighed prior to preparation of cytosol.

In addition to the identified possible biochemical effects of variable tissue dilution, it is suggested that the amount of biological material homogenised may influence NOS activity. Nematocyte discharge can be induced by mechanical stimulation (Shick, 1991) and the homogenisation technique used in these studies resulted in the discharge of nematocytes (personal observation). The degree of mechanical stimulation applied to tissue preparations during homogenisation is likely to be influenced by the volume of tissue homogenised and as a result has potential to result in different amounts of nematocyte discharge. NO has been shown to be involved in nematocyte discharge in the species *A. diaphana* (Salleo *et al.*, 1996). It is therefore possible that the determined levels of NOS activity may have been influenced by homogenisation induced nematocyte discharge. Further work is required to (a) investigate the potential involvement of NO in the discharge of nematocysts in *A. pallida*, and (b) to quantify the possible contribution of nematocyte discharge to overall NOS activity.

In addition to potential variance in NOS activity due to dilution of biological preparations, some variance may be due to the particular environmental conditions the anemones were exposed to. The lower level of variance in NOS activity observed within, and between long term incubator maintained anemones compared to recently collected animals is likely to reflect the higher degree of homogeneity of the incubator environment relative to the natural environment. Ambient conditions experienced by organisms in the natural habitat differ from those maintained in the laboratory in numerous way including differences in: thermal

regime, nutrient availability and levels of illumination and photoperiod. Dissolved nutrient concentrations at Walsingham Pond are much higher than those present in LNSW (Muller-Parker *et al.*, 1990) and the nutritional status of both host and zooxanthellae might be expected to differ as a result. In addition to being influenced by their own physiological processes, the biochemistry of host tissues of *A. pallida* are likely to be influenced by the resident symbiotic zooxanthellae as has been shown to be the case with the symbiotic anemone *Anthopleura elegantissima* (Weis and Levine, 1995). The substrate of the NOS catalysed reaction (L-arginine) functions as a nitrogen-rich storage compound in several algal taxa but zooxanthellae from anemones maintained in nitrogen poor cultures have been reported to contain very little arginine (Ferrier, 1992). *A. pallida* maintained in culture and fed brine shrimp on a biweekly basis (as the anemones used in this study were), have been shown to have big pools of arginine and to be nutrient sufficient (Ferrier, 1992). The influence of *in situ* biochemical parameters, i.e. nutritional sufficiency, photosynthetic rate, respiratory rate of both host and zooxanthellae, reproductive status of both host and zooxanthellae, on levels of NOS activity has not yet been observed but might have implications for, and account for some of the variability observed in levels of NOS activity in host preparations. Future work should aim to standardize ambient conditions of experimental animals prior to and during experiments.

Identification of potential differences in ambient conditions laboratory maintained and wild anemones are exposed to indicate that acclimatory changes are likely in organisms transplanted from the field into the laboratory. Potential acclimation, and its influence on NOS are subjects of interest for future research. The high variance in levels of enzyme activity observed in recently collected anemones might reflect variations in the microenvironments of natural habitats. Differences in activity levels observed between sampling times may be a result of seasonal effects. Marine organisms have been noted in the past to be highly seasonal in terms of their basic physiology (Sheehan and Power, 1999)

and variations in the nutritional status have been identified in zooxanthellae isolated from *A. pallida* from the same site as those used in this study (Cook *et al.*, 1992).

Anemones used in this research were not maintained in axenic conditions. NO has been implicated in the immune responses of invertebrates (including Ottaviani *et al.*, 1993; Moncada *et al.*, 1991; Franchini *et al.*, 1995b and Zhao *et al.*, 1997), it is therefore possible that at least part of the NOS activity detected in anemone preparations reflect immunologically induced activity. If this was to be proven, some of the variance in NOS activity levels observed may reflect variations in the degree of immune stimulation of the anemones.

Bacterial colonies have been observed within the tentacles of the anemone *A. pallida* (McKinstry *et al.*, 1989; Palincsar *et al.*, 1989). In addition to being a potential stimulus of NO production, bacterial cells themselves are potential sources of NOS activity. NADPHd staining revealed structures identified as bacterial aggregates in the tentacles of *A. pallida* to possess NOS activity (further detailed in chapter 4). The possibility that these cells contributed to overall levels of NOS detected in cytosolic preparations warrants further investigation. As a result of the high variance noted, wherever possible, cytosolic preparations from a single preparation were used in experiments. In experiments in which comparisons were made between anemones subject to different treatments, anemones used had an identical collection and maintenance histories, and multiple anemones were exposed to each experimental condition. The variance noted in NOS activity suggests that the enzyme is responsive to, or affected by, environmental parameters and/or biochemical status. The fact that NOS activity appears to vary greatly with an animal's environmental history suggests that it has potential as an indicator of organismal changes occurring in response to environmental stress. However, in order for that potential to be fulfilled, a much more comprehensive understanding of the effect of baseline biochemical status of an organism in its NOS activity is required.

Enzyme kinetics

Substrate kinetics

Citrulline production was shown to be linear up to exogenous substrate concentration of 100 μM . In this regard, addition of 20 μM L-arginine in standard assays appeared to be adequate for use with cytosolic preparations of *A. pallida*. Maximum rates of NOS activity shown in *A. pallida* NOS (10.3 ± 0.42 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$ in the presence of 300 μM exogenous L-arginine) far exceed those determined using NOS from arthropod haemocytes (0.17 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$) in the presence of 300 μM exogenous L-arginine) (Radomski *et al.*, 1991). Endogenous arginine was left intact in biological preparations of *A. pallida* but was removed from material used by Radomski *et al.*, 1991, this discrepancy may partly account for the higher activity seen in anemone tissues.

Results of a study conducted at an early stage of my thesis research to obtain values K_m and V_{max} were published (Morrall *et al.*, 1998). Different results ($K_m = 110$ μM and $V_{max} = 13.0$ pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$) were obtained compared to those reported here ($K_m = 132.9$ μM and $V_{max} = 17.7$ pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$). A small concentration range of substrate was used (0.002-0.2 μM) in this preliminary published study and further the ratio of labelled to unlabelled arginine was not constant over the concentration range used. For these reasons, the results reported in this thesis are more accurate than the initial published values but are nevertheless results from a single experiment. As such, confidence in the data is limited. Three replicate experiments, using an optimised assay, and subsequent kinetic parameter determinations from the generated data are required for full confidence in the results. The V_{max} value reported here (17.68 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$) indicates that use of an exogenous substrate concentration of 20 μM was potentially limiting. This regrettably is likely to have influenced all of the citrulline assay results documented in this thesis. Use of

exogenous substrate concentrations of at least 60 μM is recommended in future studies to ensure its presence in excess.

The apparent V_{max} value obtained from *A. pallida* NOS was similar to other reports at 17.7 pmoles $\mu\text{g protein}^{-1} \text{ min}^{-1}$ (V_{max} equivalent to 15.3 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ in bovine aortic endothelial cells), (Pollock *et al.*, 1991). The apparent K_m of 132.9 μM calculated for *A. pallida* NOS is much higher than previously reported values for invertebrate and bacterial isoforms (2.5 μM and 8.2 μM respectively) (Conte and Ottaviani, 1995; Chen and Rosazza, 1994). Further work is needed to elucidate the significance of this finding but weak binding of substrate-enzyme binding in *A. pallida* cytosol is suggested.

Time course of activity

While rates of citrulline formation in *A. pallida* cytosol was found to be linear only up to 10 minutes incubation time, 30-minute assay incubation times were maintained to ensure consistency and allow comparison between all experimental data. The possible problems that result from predicting enzyme activity from outside the linear, zero-order reaction period was not appreciated until after the research was completed. This realisation has implications for the entire citrulline assay data set presented in this thesis. Incubation times should have been adjusted to ensure enzyme activities were always determined within the zero-order reaction period. Future work must ensure enzyme activities are predicted from within the zero-order reaction period.

Enzyme activity in relation to biological material

Volumes of 10 μl cytosolic preparation contained detectable levels of enzyme activity and were used in standard assay incubations. Rates of enzyme activity in relation to protein concentration were found to vary between preparations. For example, enzyme activity was

found to be linear up to approximately 25 μg protein in one study (Morrall *et al.*, 2000) whereas data presented here indicated linearity is maintained only up to approximately 11.1 μg protein. It is essential that future studies ensure that rates of enzyme activity are always determined in the linear range of protein concentrations. Investigating enzyme activity linearity in a large number of preparations and using the results to determine an appropriate assay protein concentration to use as a standard may achieve this.

Effects of treatment with exogenous arginine

The results shown here illustrate reduced citrulline formation in cytosolic preparations incubated with an excess unlabelled substrate and are thus in agreement with those of Colasanti *et al.*, (1995). The reduced detected levels of citrulline formation is likely to be a result of out-competition of the labelled arginine by a much higher concentration of unlabelled substrate. Results showing very similar levels of NOS activity in preparations incubated either with, or without exogenous unlabelled arginine provide evidence that addition of standard concentrations of exogenous unlabelled arginine is not sufficient to alter detected levels of citrulline activity by out competition of the labelled substrate.

Effects of treatment with exogenous cofactors

Results from studies showing that NOS activity was not increased by addition of exogenous cofactors suggest that endogenous cofactors are present in cytosolic preparations. Addition of exogenous cofactors was shown to be unnecessary by these findings. NOS activity levels were shown to increase in response to addition of exogenous NADPH suggesting dependence on this cofactor, however these findings were not supported by the results of low molecular weight exclusion studies. NADPH dependence has been shown in a variety of NOS isoforms including those from invertebrates (Radomski *et al.*, 1991; Colasanti *et*

al., 1995) and further work is suggested to establish the importance of NADPH in the activity of *A. pallida* NOS.

Partial purification of NOS, by removal of the low molecular weight components resulted in increased enzyme activity suggesting the presence of interfering cytosolic components in crude preparations. These findings contrast with those of (Radomski *et al.*, 1991) that show NO formation to be greatly reduced in haemocyte cytosol from horseshoe crabs from which the low molecular mass components had been removed by G-25 chromatography. These findings suggest that the affinity of cofactors for NOS may be different in these species. The lack of response to addition of exogenous cofactors to G25 eluant shown in these studies has been previously noted and suggested to be due to endogenous cofactors remaining in the eluant owing to a high affinity for NOS (Franchini *et al.*, 1995b). It is recommended that G-25 chromatography of cnidarian tissue preparations is used prior to future citrulline assays in order to eliminate potentially interfering cytosolic components and hence provide a more consistent preparation.

Addition of CaCl_2 to partially purified cytosolic preparations resulted in a reduction of enzyme activity, a finding previously shown in an invertebrate study (Radomski *et al.*, 1991). Cnidarian NOS has however previously been reported to be calcium dependent (Salleo *et al.*, 1996; Colasanti *et al.*, 1997; Colasanti *et al.*, 1995). Owing to the reported tight binding of some cofactors to NOS, and the interference by endogenous cofactors in crude preparations, studies with highly purified enzyme with specific cofactor inhibitors are suggested to enable accurate determination of the cofactor requirements of NOS.

Effects of treatment with arginine analogues

Cytosolic preparations of *A. pallida* incubated with concentrations of L-NAME and L-NNA up to 3 mM did not cause significant inhibition of NOS activity. These findings contrast with those of Salleo *et al.*, (1996) who reported a significant decrease in the rate of citrulline

formation in acontial preparations from the anemone *A. diaphana* incubated in 1 mM L-NNA or L-NAME (reductions in activity reported: 37% and 34% respectively compared to formation in controls). Use of different biological preparations, different incubation periods and temperatures in these two studies may account partly for the different results. The apparently lower sensitivity of *A. pallida* cytosolic NOS to L-NAME and L-NNA compared to the effects shown in acontial preparations of *A. diaphana* is surprising. It is possible that use of cytosolic preparations of whole anemones may have masked any low levels of inhibition of NOS activity.

Incubation of cytosolic preparations of *A. pallida* incubated with 5 mM L-NNA resulted in statistically significant reductions in rates of citrulline formation suggesting that L-NNA has an inhibitory effect on NOS activity. Inhibition of NOS activity was shown in samples with intact, or removed endogenous arginine indicating that the presence of endogenous substrate does not influence the inhibitor effect of this arginine analogue. The reduction of NOS activity in samples treated with 1 mM D-NMA suggests that the NOS activity was affected partly by non-specific mechanisms. L-NMA proved to be a more potent inhibitor of NOS activity in *A. pallida* preparations than both L-NAME and L-NNA. Biological effects due to arginine analogues are limited by rates of membrane transport and studies of uptake kinetics suggest that there are differences between the various arginine analogues (Schmidt *et al.*, 1995). Uptake of L-NNA and L-NMA have been reported to be selectively inhibited by distinct amino acids (Schmidt *et al.*, 1995) indicating that the biochemical status of the organism or biological preparation may influence the inhibitory effect observed. Differences in uptake kinetics may explain the observed differences in inhibitory effects of the arginine analogues tested.

Formation of ^3H -citrulline was inhibited by L-NMA in a dose-dependent manner, but residual activity remained even in the presence of high concentrations of L-NMA. These

findings contrast with the complete inhibition by L- NMMA of NOS activities reported in mammalian macrophages and platelets (Hibbs *et al.*, 1990; Radomski *et al.*, 1990), but are in agreement with results from a number of studies of invertebrate preparations (Radomski *et al.*, 1991; Elphick *et al.*, 1993; Johansson and Carlberg, 1995). A multitude of reasons have been suggested for the lack of complete inhibition of invertebrate NOS by arginine analogues. Among these are: metabolic pathways distinct from that catalysed by NOS may be present that lead to L-citrulline formation from L-arginine (Giraldez and Zweier, 1998), L-NMA may be metabolised by NOS (Klatt *et al.*, 1994) and it has been suggested that conversion of L-NMA to citrulline and NO may occur more rapidly *in vivo* than it does in isolated enzyme studies *in vitro* (Griffith and Gross, 1996), finally, the ratio of L-arginine: L-NMA in the vicinity of NOS may be much higher than would be predicted from extracellular concentrations (Griffith and Gross, 1996). In addition to these suggestions, it is likely that differences in NOS inhibition characteristics seen between species represent differences between mammalian and invertebrate NOS isoforms (Radomski *et al.*, 1991). Phylogenetic evidence indicates that the gene duplication preceding the evolution of an immunological role for iNOS in mammalian species may have occurred after the divergence of vertebrates and invertebrates (Hughes, 1998). Diversity and regulation would then have arisen convergently in these groups and may account for the different characteristics of isoforms from different groups of organisms.

The K_i determined for *A. pallida* (1014 μM) is far in excess of previously determined values, which range from 0.18-7.4 μM (Griffith and Gross, 1996). The high value determined from this study is likely to be due to the unpurified nature of the enzyme preparation used for this determination; purified enzymes are typically used in K_i determinations.

Overview of arginine analogue effects

Arginine analogues were shown to inhibit the activity of *A. pallida* NOS. L-NMA was the most potent NOS inhibitor tested. Incomplete inhibition supports suggestions that NOS isoforms present in vertebrate and invertebrates differ. Use of L-NMA is suggested for future work with cnidarian NOS, including in studies designed to elucidate the role(s) played by NO in the biology of these organisms.

Suggestions for further work with arginine analogues

Investigations into the specificity and reversibility of the inhibitory effect of arginine analogues on cnidarian NOS to arginine analogues are recommended by comparative studies of the effects of inactive and active enantiomers, and administration of arginine analogues along with an excess of substrate. Previous studies of cnidarian NOS have showed enzyme inhibition due to L-NAME and L-NNA to be reversible and D-NAME has been shown to be inactive (Colasanti *et al.*, 1997; Salleo *et al.*, 1996). Also worthy of further study is investigation of the extent of potential interaction of arginine analogues and exogenous substrate by comparison of NOS inhibition in biological preparations incubated with arginine analogues with and without addition of unlabelled exogenous arginine. Purification of *A. pallida* NOS would enable determination of a precise K_i value and comparison with the value obtained by these studies would enable the extent of the interference from the complex biochemical matrix of the crude preparation to be quantified. The significance of the potential contribution to NOS activity of nematocyte discharge warrants investigation, including studies using arginine analogues.

Effects of treatment with an exogenous NO donor

Cytosolic incubation

Reduced levels of citrulline formation noted in anemones and cytosolic preparations incubated with SNP provides evidence of inhibition of NOS activity by exogenous NO production. Inhibition or down regulation of NOS activity in response to NO has been widely suggested and documented (Hecker and Billiar, 1996; Griscavage *et al.*, 1994; Giovanelli *et al.*, 1994; Stuehr and Griffith, 1992; Knowles and Moncada, 1994). NO inhibits NOS activity in both purified and unpurified preparations of NOS of a variety of types from a variety of sources; this inhibitory activity appears to be due to NO itself rather than to a chemically related species (Griscavage *et al.*, 1994). NO exerts feedback inhibition of NOS activity probably by interacting with the enzyme's heme prosthetic group (Franchini *et al.*, 1995b). Further work with potassium ferrocyanide, an analogue of sodium nitroprusside that does not release NO (Delledonne *et al.*, 1998) is recommended to determine if the effect seen with SNP was NO specific.

Effects of lipopolysaccharide on NOS activity

Owing to inconsistencies in the results of the pilot studies documented here, no conclusions can be drawn on the response of *A. pallida* to LPS. The variable responses observed may be due to variable degrees of immune stimulation present in control anemones resulting from their maintenance in non-axenic conditions. The presence of variable amounts of naturally occurring immune stimuli that could interact with exogenously applied LPS may account for the variable results. It is also possible that stimulation of NOS activity by LPS was not detected owing to a dilution effect resulting from preparation of whole anemone tissue. Phagocytes (cellular structures responsible for part of the non-specific immune response in invertebrates) have been previously reported to occur in the mesoglea of sea anemones in low numbers, while aggregations of them make up the mesenteric filaments at the base of

some anthozoan species coelenterons (Smith and Hutton, 1995). It would be of great interest to measure NOS activity in aggregations of *A. pallida* phagocytes (prepared as outlined in the methods of Smith and Hutton, 1995) exposed to LPS to better clarify the inducible status of NOS in this species. Future experimental design should consider the suggestion of Franchini *et al.*, (1995b) that LPS is not the most effective inducer of immune responses and that greater responses have been found when two stimulating compounds are combined.

CHAPTER FOUR

LOCALISATION OF

NITRIC OXIDE

SYNTHASE

Chapter Four

Localisation of Nitric Oxide Synthase

4.1 Introduction

Both biochemical and histochemical experiments were conducted to investigate the location of NOS activity in *A. pallida*.

Soluble versus whole host fraction

Rates of citrulline formation were compared in soluble cytosolic and whole homogenate preparations from a single stock of *A. pallida* tissue using the citrulline assay (section 2.2).

The NADPH-diaphorase assay

NADPH-diaphorase (NADPHd) staining was performed on preparations of *A. pallida* to localise NOS activity. The NADPHd technique is accepted as a reliable marker for the detection of multiple NOS isoforms (Weinberg *et al.*, 1997) and has been used with a variety of different biological preparations from a range of species. Diaphorases are a group of redox enzymes that can use a reduced cofactor to reduce various chromogens, e.g. nitroblue tetrazolium, producing a coloured product. In NADPH diaphorase (NADPHd) staining, nitroblue tetrazolium (NBT) is reduced by NOS to its water insoluble form, blue diformazan (figure 4.1) that is resistant to subsequent re-oxidation (Weinberg *et al.*, 1997). Diformazan is used in histochemical preparations to localise NOS activity. Whilst not all NADPHd activity is due to NOS (Matsumoto *et al.*, 1993), NOS activity has been shown to be remarkably resistant to various protein fixatives, distinguishing it from other known NBT-reducing enzymes (Weinberg *et al.*, 1997). As a result, fixation of tissue in aldehyde increases the specificity of the stain and enables convenient histochemical localisation of NOS.

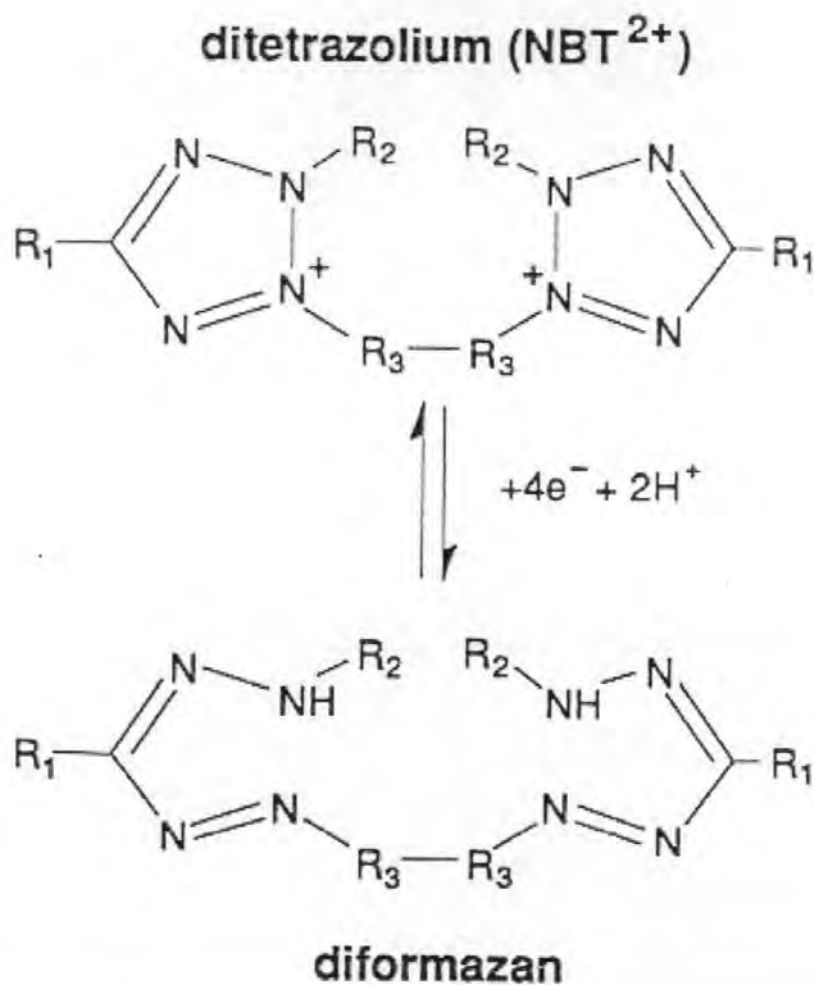


Figure 4.1. The NADPHd reaction. NOS catalyses the NADPH-dependent reaction of nitroblue tetrazolium (NBT) to an insoluble diformazan. $R_1 = \text{C}_6\text{H}_5$, $R_2 = \text{p-NO}_2\text{C}_6\text{H}_4$, and $R_3 = \text{m-CH}_3\text{OC}_6\text{H}_4$. From Weinberg *et al.*, (1997).

4.2 Materials and methods

Biochemical localisation

Anemones were homogenised (as detailed in section 2.1) and the resulting homogenate split into two equal size aliquots. One aliquot was held on ice whilst the other was centrifuged (10 minutes x 16000 RCF) at 4°C to separate zooxanthellae and insoluble host material from the soluble (cytosolic) fraction. Standard citrulline assays were performed on whole homogenate and the cytosolic fraction both with and without addition of exogenous cofactors and the resulting enzyme activities were compared.

The NADPHd assay

Triton X-100 was obtained from BioRad (USA). The remaining chemicals including (2,2'-Di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-[3,3'-dimethoxy-4, 4'-diphenylene] ditetrazolium chloride), nitro blue tetrazolium (NBT) and Dicumarol were obtained from Sigma (UK). Sections were made with a Leica microtome.

The following solutions were prepared: anaesthetising solution (0.2 M MgCl₂), Tris buffer (TBS), made up with a mix of A (0.5 M Tris [hydroxymethyl] aminomethane hydrochloride) and B (0.5 M Tris [hydroxymethyl] aminomethane). TBS was prepared by adding tris B to 150 ml tris A until a pH of 8 was reached. Phosphate buffer (PBS) was prepared by mixing 13 ml 1 M mono sodium phosphate (solution requires heating for complete dissolution) and 87 ml 1 M disodium hydrogen phosphate and adjusting the pH to 7.6. An incubation solution was made up with 0.5 mM NBT, 0.1 mM dicumarol, 1 mM NADPH and 0.25% Triton-X in TBS, with a pH adjusted to 7.0-7.1. An 8% stock of paraformaldehyde was made up in deionised water. The solution was heated and stirred at 60°C for 1.5 hours, then at 80°C until complete dissolution occurred. Paraformaldehyde stock was stored at 4°C in a screw top glass vessel capped with foil. Glutaraldehyde was dissolved in deionised water. Three different fixative solutions were made up: 4%

paraformaldehyde & 2.5% glutaraldehyde, 4% paraformaldehyde & 0.5% glutaraldehyde and 4% paraformaldehyde. Fixative mixtures were prepared in PBS and deionised water. Fixative solutions were cleared with 1 M NaOH.

The assay

Principal steps

1. Fixation.
2. Staining.
3. Embedding.
4. Sectioning.
5. Examination

Whole anemones were anaesthetised in $MgCl_2$ for 10 min to achieve body and tentacular relaxation, then rinsed thoroughly with seawater to remove residual $MgCl_2$. Fixation was achieved by immersing anemones for 1 hour at 18°C in one of the three fix solutions. Post fixation anemones were well rinsed with PBS (3 x 10 minutes) (a significant reduction in the intensity of the stain has been shown to occur when any fixative remains in the preparation).

Staining and tissue clearing

Anemones were divided in two longitudinally and the sections immersed in freshly prepared incubation solution. Control incubation solutions were prepared without addition of NADPH. After dark incubation at 18°C for 16 h, anemones were rinsed with TBS, immersed in 100% glycerol followed by 70% ethanol to clear tissues. A 21 h automated tissue-processing programme (Shandon Hypercentre 2, program detailed in appendix 2) prepared the tissue for embedding and sectioning.

In order to observe possible NOS production induced by exposure to lipopolysaccharide (LPS) (further details on LPS are given in chapter 3), whole anemones were pre-treated with LPS 24 hours prior to NADPHd staining. Anemones were injected with 20 µg LPS, or immersed in 20 µg/ml LPS solution. Resulting staining patterns were compared with those in anemones not exposed to LPS.

Embedding

Brass moulds (1 x 2 cm) were prepared on metal plates. Moulds were filled with molten wax. Labels were embedded at one end of the mould. Stained animals were oriented in the wax. A skin was formed on the wax by gently blowing onto the surface; the block was then lowered slowly (to ensure the wax set without crystallising) into a water bath at room temperature. After a few minutes immersion, brass moulds were removed; the wax block was then roughly levelled with a fixed blade scalpel then mounted onto a wooden block. The wax block sides were straightened by gentle melting prior to sectioning.

Wooden blocks with attached wax blocks were secured in the microtome clamp. Sections of between 5-20 µM were cut and transferred with a fine paintbrush onto the surface of a 30% alcohol solution momentarily (to displace any air) before floating on a water bath at 55°C. Sections were then transferred onto glass slides that were pre-treated with a thin layer of egg white. Slides were labelled with a diamond marker and dried overnight at room temperature. Slides were then cleared by immersing for 2-5 minutes in xylene; cover slips were applied with mounting solution. Slides were examined on a light microscope and the distribution of dye observed and noted. Photographs of sections were taken with an Olympus Atl. 2 mounted on an Olympus AX 70 light microscope.

4.3 Results

Location of NOS activity in preparations of *A. pallida*

Slightly higher, but not statistically significantly different rates of citrulline formation were detected in cytosolic fractions relative to whole homogenate (0.42 ± 0.07 and 0.36 ± 0.01 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ respectively). Values are combined mean and s.d. data from three separate experiments.

Histochemical localisation

Staining was optimised in tissues of *A. pallida* incubated for 3–4 hours in 4% paraformaldehyde & 2.5% glutaraldehyde in phosphate buffer fixative. NADPHd staining of anemones showed the presence of NOS enzymes in the entire epidermal layer of *A. pallida* (figure 4.2), staining was largely restricted to these tissue layers. Staining was absent in control anemone sections (figure 4.3). In both body column sections (figure 4.4) and tentacle sections (figure 4.5) cells comprising the ectodermal layer stained diffusely. In tentacle sections, a distinct band of intense stain is present at the outermost edge of the mesoglea. In body column sections, a band of similar intensity stain is visible along the inner edge of the mesoglea. In body column and tentacle sections the gastrodermis was largely unstained (figures tentacle 4.4 and 4.5). A few zooxanthellae appear stained (examples not shown). In body wall sections, the mesoglea is unstained (figure 4.4). No changes in staining patterns were observed in anemones injected with or incubated in LPS for 24 hours prior to NADPHd staining. Structures visible in the tentacle epidermis of *A. pallida* resemble those identified as bacterial aggregates by McKinstry *et al.*, (1989) and Palincsar *et al.*, (1989) in terms of location, shape and size. These structures were observed

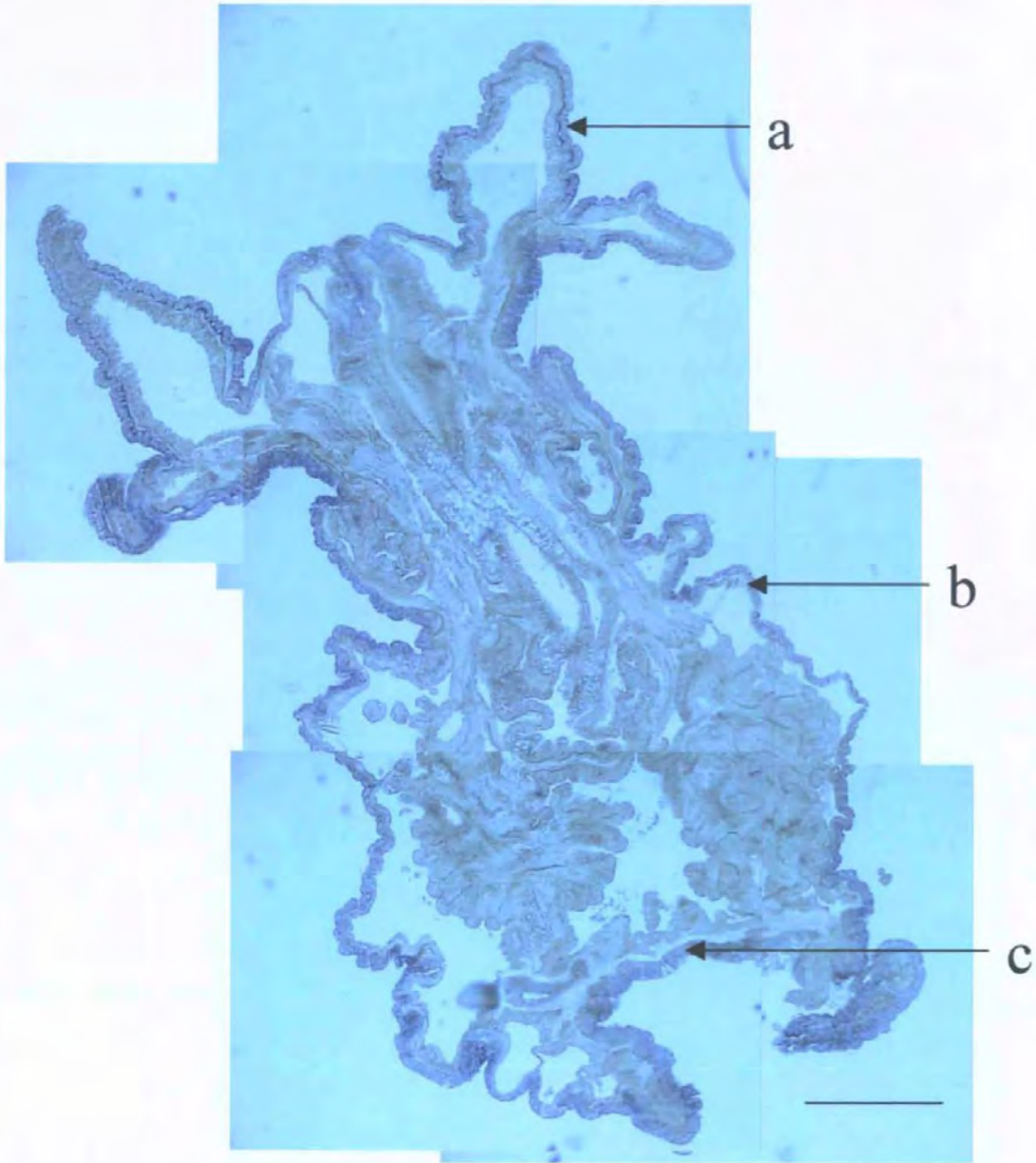


Figure 4.2. Composite image of entire anemone. Staining is visible as purple coloration in the epidermal layers of the anemone. Scale bar indicates 0.5 mm. a=tentacles, b=column, c=pedal disc.

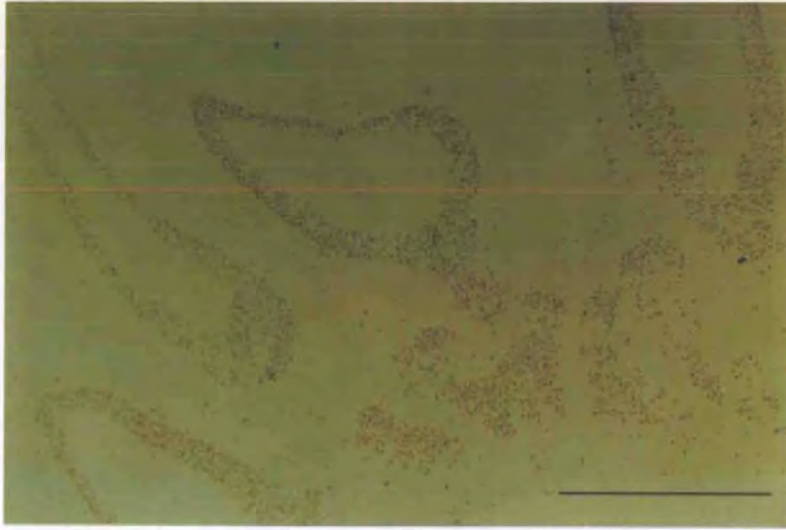


Figure 4.3. Cross section through a tentacle of *A. pallida* treated with NADPH free (control) solution. NADPHd staining is absent. Zooxanthellae are clearly visible. Scale bar indicates approximately 0.25 mm.

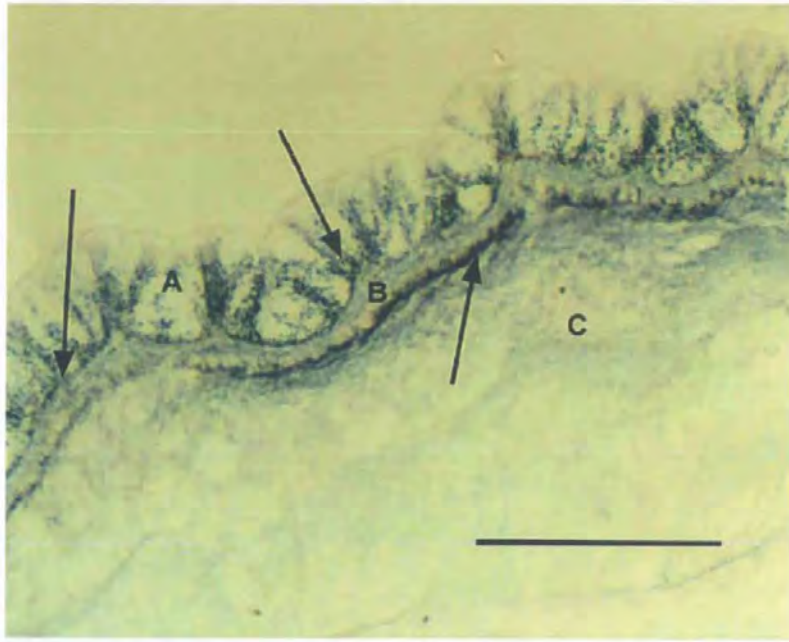


Figure 4.4. NADPHd stained cross section through anemone column. Scale bar indicates 0.15 mm. A=epidermal cell, B=mesoglea and c= gastrodermis.

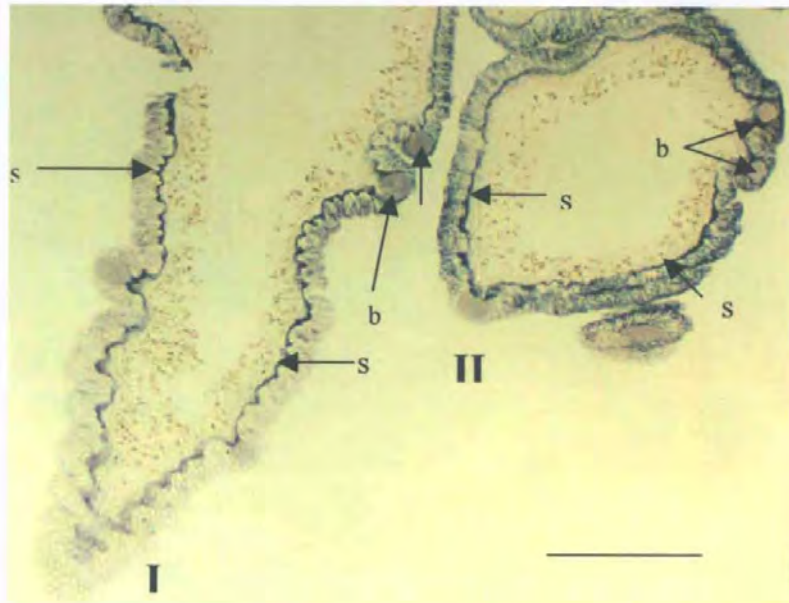


Figure 4.5. NADPHd staining of tentacle sections. I= longitudinal section, II= cross section. Scale bar indicates 0.25 mm. s= indicates appearance of purple/blue diformazan stain, b= bacterial colonies.

to be lightly and evenly stained (figure 4.5). These findings contrast with a reported absence of aggregates in *A. pallida* from Bermuda (Palincsar *et al.*, 1989). These authors do however note bacterial aggregates associated with *A. pallida* from a range of locations to be most common at tentacles tips, but they were also observed proximally on the tentacles, on the oral disc, and at low density throughout the column. In this study, bacterial aggregates were observed only in anemones tentacles.

4.4 Discussion

Comparison of soluble and whole homogenate preparations

Preparation of the cytosolic fraction from whole homogenate removes insoluble material, including the insoluble protein fraction. Results showing higher levels of NOS activity in cytosolic preparations than in whole homogenate samples illustrate that NOS enzymes from *A. pallida* are contained in the soluble fraction. These findings are in line with those of Matsumoto *et al.*, (1993) that showed more NOS activity in the cytosolic fraction than in the particulate fraction in eight different sections of rat brain, but contrast with those of Giraldez and Zweier (1998) who found NOS activity to be mostly associated with the particulate fraction of rat heart preparations. These contradictory findings illustrate the existence of differences in location of NOS enzymes in different tissues. Future work is needed using a sub-cellular fractionation technique (i.e. using centrifugation to isolate specific cellular organelles and/ or components based on differences in size, shape or density) to enable specific localisation of the NOS enzyme in *A. pallida* preparations. An isotonic homogenisation buffer (such as HB with sucrose or salts added) should be used for tissue preparation prior to fractionation in order to maintain intact cellular organelles.

NOS localisation by NADPHd staining

Occurrence of false positive staining was ruled out by the absence of diformazan stain in control tissue sections incubated in NADPH free staining solution. Staining was optimised in *A. pallida* tissues incubated for 3-4 hours in 4% paraformaldehyde, 2.5% glutaraldehyde in phosphate buffer fixative. Patterns of diformazan staining indicating the presence of NOS activity were related to anthozoan biology with reference to the comprehensive study of Hyman (1940). The distribution of NOS activity throughout the outer epithelial layer and in cells adjacent to the inner and outer edges of the mesoglea shown by NADPHd staining is compatible with a broad involvement of NOS in both sensory and motor activities. Sensory cells are very numerous in tentacles and may account for the intense band of staining visible at the base of the ectoderm. The epidermal nerve cells are located near the mesoglea. The intense band of stain seen between the inner edge of the mesoglea and the gastrodermis in body column sections may be that of NOS located in circular gastrodermal muscle. It is unclear why staining was associated with a few zooxanthellae cells; further investigations are required to reveal the significance of this finding.

A previous study has implicated NO in the discharge of nematocysts and identified NOS in the supporting cells between the apical parts of adjacent nematocytes in acontial preparations from *A. diaphana* (Salleo *et al.*, 1996). No staining was evident in acontia in *A. pallida*. Further work is necessary to show if this is due to variance of experimental techniques (i.e. use of acontial preparations rather than entire anemone sections), or due to a biological difference between species. Elofsson *et al.*, (1993), using essentially similar techniques to those used here, found only non-selective staining in *Aiptasia* sp. and in the freshwater cnidarian *Hydra oligactis*. These authors suggested that the distribution of NADPHd positive cells might be restricted to invertebrates having well developed nervous systems. Cnidarians possess a simple nervous system, yet the results shown here indicate specific staining. These results therefore contrast to the findings of Elofsson *et al.*, (1993).

Anemone tentacles have a high surface area to volume ratio and are present in large numbers, thus the tentacles of *A. pallida* represent a large proportion of the anemones epidermal layer. These studies showed NOS activity to be associated almost exclusively with the epidermal layers and as a result it is likely that the majority of anemone NOS is present in the tentacles. The role played by NO generated in anemone tentacles is not known, but may be associated with the chemosensory function of the structures.

The light and even staining observed in structures identified as bacterial colonies present in tentacle epidermis indicate NOS activity. As these bacterial colonies were included in homogenate and cytosolic preparations of *A. pallida*, it is probable that bacterial NOS activity contributed to overall levels of NOS detected in preparations. Further work is required to investigate the significance of bacterial NOS in *A. pallida*. Palincsar *et al.*, (1989) report that examination of a range of *A. pallida* from a range of locations revealed the highest numbers of bacteria to be contained in recently collected anemones from North Carolina, while no bacterial aggregates were reported in anemones from Bermuda. Bacteria noted to be associated with *A. pallida* have been found to be gram-negative, and it has been suggested that they may belong to the genus *Vibrio* (Palincsar *et al.*, 1989). Bacteria have been noted to occur in a range of coral species (Peters *et al.*, 1983; Peters 1984). Single bacteria cells have often been observed as endosymbionts in cells of many types of organisms (Palincsar *et al.*, 1989).

The absence of alteration of staining pattern observed in anemones pre-incubated in LPS suggests that *A. pallida* NOS is not inducible. Of note is the complete absence of staining in the mesenteric filaments that usually contain high numbers of immunocytes (Smith and Hutton, 1995). These findings, along with those detailed in chapter 3 that showed no change in NOS levels in anemones treated with LPS, indicate that the NOS present in *A. pallida* is a constitutive rather than inducible isoform. These findings do not support the hypothesis that NOS has potential as a biochemical effect biomarker in *A. pallida*.

An accumulating number of data implicate NO as an intercellular messenger in invertebrate tissues, including the nervous system, excretion organs, salivary glands, haemocytes, immunocytes and endocrine cells (Johansson and Carlberg, 1995). The presence of an NO signalling system in such a variety of tissues suggests multiple roles for NO in invertebrates (Johansson and Carlberg, 1995). In many invertebrate species in which NOS has been studied, the enzyme appears in the central and peripheral nervous system. In molluscs, there has been an apparent evolution in the involvement of NO in muscle cell physiology (Martinez, 1995). The more primitive groups such as the polyplacophora show NADPHd staining only in buccal muscle cells. In other phyla, positivity is apparent in the neurons innervating these muscles, which may indicate a switch in the site of NO-mediated control of muscle motility. NOS activity has been shown to be a controlling factor in the primitive olfactory feeding response of the cnidarian *Hydra vulgaris*, considered the most primitive multicellular organism to possess a nervous system (Colasanti *et al.*, 1997). The response consists of tentacular writhing and mouth opening and is similar to the feeding response seen in *A. pallida*. Involvement of NO in the regulation of smooth muscle tone has been demonstrated in the echinoderm *Asterias rubens* (Elphick and Melarange, 1998) in which relaxation of the cardiac stomach was induced using the NO-donor chemicals S-nitrosoglutathione (SNOG) and S-nitroso-N-acetylpenicillamine (SNAP).

Recommendations for further work

The results from NADPHd staining presented here are those of a preliminary study using halved section of anemones. The pattern of staining observed may have been influenced by the penetration of the staining chemicals into tissues. Despite this, the results provide a good indication of the principal localisation of NOS in *A. pallida*. Staining of pre-prepared fixed and sectioned tissue is recommended for future work. Immunocytochemical methods are recommended to verify the findings of NADPHd staining (as suggested by Johansson and

Carlberg, 1995), however interpretation of results obtained from invertebrate studies needs careful consideration. To date specific invertebrate NOS antisera are unavailable, necessitating use of antisera raised against mammals; NOS isoforms may be present in invertebrates that are not recognisable by mammalian NOS antisera (Johansson and Carlberg, 1995).

Conclusions

Findings of this research indicate that *A. pallida* NOS is associated with the soluble fraction of whole homogenate preparations. The presence of NOS activity was demonstrated in the outer epithelial layer and adjacent to the inner and outer edges of the mesoglea and it is speculated that this distribution is compatible with NOS involvement in sensory and motor activities. The lack of change in NADPHd staining in LPS treated animals indicates that NOS in *A. pallida* is a non-inducible isoform. As such, the potential usefulness of the enzyme as an exposure and/or effect biomarker is questionable.

CHAPTER FIVE
RESPONSES OF
***A. pallida* TO**
CONTAMINANT
EXPOSURE

Chapter Five

Responses of *A. pallida* to Contaminant Exposure

5.1 Introduction

Tropical ecosystems are under increasing threat of development and habitat degradation from population growth and urbanisation, agricultural expansion, deforestation, and mining (Lacher and Goldstein, 1997). Species belonging to the phylum Cnidaria dominate the shallow marine environments of the tropics and subtropics. Many aspects of cnidarian biology suggest that they are useful test organisms for toxicological studies (further detailed in sections 1.6 and 1.8). The predominantly sessile lifestyle, high surface area to volume ratio and soft-bodied form of cnidarian species results in them being in intimate contact with their environment. As a result, cnidarians may be acutely vulnerable to water borne contaminants. Symbiotic associations between cnidaria and zooxanthellae are common in the tropics and subtropics. Despite increasing knowledge of the dynamics and sensitivity of symbiotic relations, toxicological studies on these associations are scarce (Mercier *et al.*, 1996).

Biomarkers are now generally accepted as useful tools in monitoring programs that assess the impact of pollutants and anthropogenic activities on marine organisms (Kushmaro *et al.*, 1997). In addition, biomarkers that are responsive to chemical challenges (effect biomarkers) can be used to explore the mechanisms involved in contaminant-induced alterations (Lowe *et al.*, 1995a). Alterations in biochemical systems are often more sensitive indicators than those at higher levels of organisation (Stegeman *et al.*, 1992) and detection of changes at this level may provide the most sensitive and earliest indication of contaminant induced effects.

NO has been shown to be involved in a huge number and range of physiological and pathological roles, in a wide diversity of organisms (for review see Nathan and Xie, 1994).

The roles played by NO in cnidarian species are largely undocumented, but the demonstrated functional importance of NO in other invertebrate species suggests that it is likely that NO is essential to the species of this phylum. It has been hypothesised that NO, together with an array of highly conserved molecular mediators including cytokines and neuroactive molecules, co-ordinate the responses of invertebrate haemocytes to a variety of stressors (Ottaviani and Franceschi, 1997). Whilst the majority of published data relating to this theory has been accumulated in other invertebrate phyla, predominantly in the mollusca, the ancient origins of many of the molecules mentioned, spanning phyla separated by 500 million years of evolution, suggest such fundamental processes might reasonably occur in other invertebrate phyla. It is reasonable to propose therefore, that NO might play a role in the stress response of *A. pallida*. Despite recognition of the importance of NO in invertebrate stress responses, the effects of chronic pollution on NO production and hence the activity of the enzyme responsible for its generation (NOS) has to date been largely unexplored. NOS enzymes are large and complex and use several cofactors and substrates to catalyse a multi-stage oxidation reaction. As such NOS enzymes are vulnerable to the effects of xenobiotics through potential interactions with substrate and cofactor binding sites or through alterations in cellular energy balance and the relative concentration of cellular constituents.

Experiments were designed to test the hypothesis that NOS has potential to be an exposure biomarker in *A. pallida* to a range of contaminants. NOS activity was determined in *A. pallida* exposed to a range of contaminants. Owing to the unprecedented nature of this study, a previously validated biomarker and a widely reported cnidarian stress response were monitored alongside NOS activity in contaminant-exposed anemones. These took the form of lysosomal stability assessment via the neutral red assay (detailed in section 2.5) and morphological status monitoring. Lysosomal stability is a well-validated biomarker of cellular stress (Ringwood *et al.*, 1998) and morphological changes have been noted in a

range of cnidarian species in response to a variety of contaminants (Black *et al.*, 1995; Blaise and Kusui, 1997; Trottier *et al.*, 1997; Harland and Nganro, 1990; Evans, 1977; Mercier *et al.*, 1996; Mercier *et al.*, 1997). The experiments designed were preliminary and no attempt was made to link contaminant effects to measures of Darwinian fitness (i.e. reproductive or growth potential).

To further knowledge of the role played by NO in *A. pallida* and to investigate possible links between NOS activity and morphological status in this species, arginine analogues and an NO donor were employed. Synthesis of NO by vascular endothelium in humans is responsible for the vascular tone essential for the regulation of blood pressure, (Moncada and Higgs, 1993). Some parallels can be drawn between blood pressure regulation in humans and regulation of the hydrostatic skeleton of anemones and experiments were designed to test the hypothesis that NO is involved in regulating the morphological status of anemones.

Rationale of contaminant choice

The poly-aromatic hydrocarbon (PAH) fluoranthene, bis (tri-n-butyltin)-oxide (TBTO) and copper were the contaminants chosen for these studies as they are widely distributed in the marine environment and have each previously been used in toxicological studies with marine invertebrates (including: Mercier *et al.*, 1996; Mercier *et al.*, 1997; Mercier *et al.*, 1998; Smith *et al.*, 2000; Bouchard *et al.*, 1999; Regoli *et al.*, 1998a; Harland and Brown, 1989; Harland *et al.*, 1990; Harland and Nganro, 1990; Kasschau *et al.*, 1980; Reichelt-Brushett and Harrison, 1999; Lowe *et al.*, 1995b; Eertman *et al.*, 1995; Long, 1992). A number of other specific reasons determined the choice of contaminants; both TBTO and copper have been used in previous studies using sea anemones and morphological changes resulting from exposure to these contaminants have been noted (Harland and Nganro, 1990; Mercier *et al.*, 1996 and 1997). Two of the studies available to date that relate contaminant

exposure to NOS activity used TBTO (Kergosein and Rice, 1998 (studies exposed intact mice to TBTO via injection and additionally exposed mice macrophages to TBTO); Smith *et al.*, 2000 (exposed intact *Mytilus edulis* in TBTO spiked seawater)). Additional support for selection of TBTO and fluoranthene in this research was provided by results of previous studies that have described organotins and PAHs as prototypic immunotoxic agents (Kergosein and Rice, 1998) and the proven importance of NO in immune responses of many organisms, including invertebrates (Ottaviani *et al.*, 1993, Ottaviani *et al.*, 1995). The neutral red assay has not previously been reported using a cnidarian species, but documentation exists on the lysosomal responses of other marine invertebrates to copper and fluoranthene (including Regoli *et al.*, 1998a; Ringwood *et al.*, 1998; Lowe *et al.*, 1995b; Lowe and Pipe, 1994 and Grundy *et al.*, 1996). Information on each of the chosen contaminants is given later in this section.

Physiological response monitoring

The degree of expansion or contraction of sea anemones and other species of cnidarian is easily visualised and quantified. Morphological changes of anemones can be rapid and have been noted to occur in anemones in response to a wide range of stimuli (Bohn, 1908b, cited in Pearse, 1974). Contraction can be provoked by any strong stimulus that might be considered unfavourable (Pearse, 1974) and morphological changes have been reported in cnidarians exposed to a wide range of adverse environmental conditions (Trottier *et al.*, 1997; Pearse, 1974; Black *et al.*, 1995; Batham and Pantin, 1953; Needler and Ross, 1958; Edmunds *et al.*, 1976; Batham and Pantin, 1950a). The physiological responses shown by the cnidarian *Hydra attenuata* have been suggested as a toxicity assessment tool (Blaise and Kusui, 1997). Morphological changes are brought about in anemones by muscular action acting on the hydrostatic skeleton (Shick, 1991). Owing to the soft-bodied nature and hydrostatic skeleton of anemones, muscle movement influences the mechanical conditions

of every muscle in the system (Batham and Pantin, 1950c). Cnidarians typically have an abundance of tentacles which, when fully extended, represent a large surface area which is intimately exposed to the ambient environment. By contracting, anemones reduce the surface area exposed to their external environment, which may serve to reduce or prevent damage caused by adverse conditions. In addition to influencing the exposed surface area, organismal contraction has implications for a variety of physiological processes; contracted anemones conserve energy, have reduced oxygen consumption (Pearse, 1974), and have a reduced prey capture capacity. In addition, contraction has implications for photosynthesis of zooxanthellae contained within cnidarian tissues by restricting the amount of light reaching algal cells. Contracted anemones are therefore likely to be at risk of nutritional deficiency, through reduced prey capture and photosynthate production (hence translocation to the host). Contraction therefore has implications for a number of Darwinian fitness parameters (Depledge, 1989) i.e. growth or reproductive potential. While contraction is a noted response of anemones to a range of contaminants, it must be noted that the degree of contraction/expansion of anemones may change in the apparent absence of stimuli, behaviour that has been described as inherent activity (Batham and Pantin, 1950b). Along with contraction, detachment from substrate is a recognised defensive behaviour of sea anemones (Edmunds *et al.*, 1976), which has been noted in contaminant responses (Mercier *et al.*, 1997).

Copper

Copper is a beneficial trace element required by all living organisms (Harland and Nganro, 1990). Copper acts as a catalyst for many enzyme systems and is important as an electron carrier in intracellular structures (Reichelt-Brushett and Harrison, 1999). In symbiotic cnidarians, copper is essential for specific biochemical processes, including the induction of superoxide dismutase (SOD) activity (Harland and Nganro, 1990). In spite of its well-

defined essential status, exposure to copper at concentrations in excess of organismal requirements has been shown to cause environmental problems (Depledge *et al.*, 1995). Elevated concentrations of metals in coastal environments are the result of both natural (i.e. volcanoes and natural weathering of rocks) and anthropogenic processes (i.e. mining, combustion of fossil fuels and the release of sewage) (Duquesne and Coll, 1995). It has been estimated that anthropogenic releases of copper are between one and three orders of magnitude higher than natural fluxes (Depledge *et al.*, 1995). Copper concentrations in seawater range from 8×10^{-4} µg/l in pristine open ocean environments, up to 29.2 µg/l at highly polluted sites (Sadiq, 1992). Surface water samples from the Great Barrier Reef region have shown mean copper concentrations to range from 0.11-0.24 µg/l (Denton and Burdon-Jones, 1986). Much research has been conducted on the routes, sources and cycling of heavy metals in the temperate climates of the world, however little information is however available for subtropical and tropical regions where different patterns of rainfall, ocean currents, prevailing winds and annual temperature cycles affect heavy metal input and persistence (Depledge *et al.*, 1995).

The toxicity of copper to marine organisms is fairly well documented (Mance, 1987) as a result of its widespread environmental occurrence resulting from both natural and anthropogenic activities. Toxicity is dependent on many factors including bioavailability and the responses of the organism to contaminant exposure (Harland and Nganro, 1990). A range of biological responses to copper have been noted in laboratory studies using species of molluscs and cnidarians. Noted responses include: reductions in lysosomal stability (Regoli *et al.*, 1998a), zooxanthellae loss (Harland and Nganro, 1990; Duquesne and Coll, 1995) increased mucus production (Thompson *et al.*, 1980; Harland and Nganro, 1990), reduced fertilisation success (Reichelt-Brushett and Harrison, 1999) and alterations in free amino acid pools (Kasschau *et al.*, 1980). Many of the toxic effects observed in marine animals exposed to high levels of heavy metals involve membrane-related processes

(Sheehan and Power, 1999). In addition to these noted responses, copper has been shown to increase the production of oxyradicals by stimulation of their formation and/or inhibiting the normal pathway by which these reactive species are usually removed (Christie and Costa, 1984). Absorption or excretion of metals by coelenterates takes place across the body surface (Harland and Nganro, 1990). Evidence of regulation of uptake, accumulation and elimination of heavy metals has been provided in both symbiotic and azooxanthellate temperate anemones (Bryan and Gibbs, 1983; Harland *et al.*, 1990).

Fluoranthene

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants (Grundy *et al.*, 1996). Fluoranthene is one of the most abundant PAHs (Forbes *et al.*, 1996) and is widely distributed (Lowe *et al.*, 1995b) in both terrestrial and marine environments. Seawater concentrations of PAHs in polluted environments of 505 $\mu\text{g/l}^{-1}$ have been detected (Madany *et al.*, 1994). Fluoranthene has a high bioaccumulation potential relative to smaller or larger PAHs (Landrum, 1989) due to its hydrophobic nature. Fluoranthene has previously been used as a model hydrocarbon (Lowe *et al.*, 1995b) in toxicity studies (Lowe *et al.*, 1995b, Grundy *et al.*, 1996, Donkin *et al.*, 1991, Eertman *et al.*, 1995, Forbes *et al.*, 1996). An extensive review of fluoranthene toxicity studies was published by Long (1992). Numerous adverse effects have been reported in organisms exposed both long and short term to fluoranthene. Those reported in *Mytilus edulis* exposed to fluoranthene include: reduced tolerance to aerial exposure, adversely affected gametogenesis, alterations in antioxidant activities (Eertman *et al.*, 1995), reduced feeding rates (Grundy *et al.*, 1996, Eertman *et al.*, 1995), and adverse effects on the cellular immune function (inhibition of phagocytosis, membrane disruption and disruption of the ability of lysosomes to take up NR) (Grundy *et al.*, 1996). While numerous toxic effects of PAHs are reported, the marine

polychaete *Capitella* sp. thrives in sediments heavily contaminated with PAHs as a result of its ability to metabolise fluoranthene (Forbes *et al.*, 1996).

Tributyltin (TBT)

The primary source of TBT in surface waters is vessels painted with TBT-containing antifouling paints (Cardwell *et al.*, 1999). While use of paints containing TBT was banned in most industrialised countries during the 1980s (Mercier *et al.*, 1998), owing to slow degradation and continued use on some vessels, organotins continue to be ubiquitous environmental contaminants (Fent, 1996). The two most common formulations of TBT are (bis (tri-n-butyltin)-oxide (TBTO), and (bis (tri-n-butyltin)-chloride (TBTCl) (Kergosein and Rice, 1998). The distribution and persistence of TBT in the marine environment is complex; TBT degrades rapidly in seawater where it has a residence time of days only, but as it tends to adsorb on to particles and aggregate in sediments, the degradation processes are considerably slower (Evans, 1999). As a result the half-life of TBT in sediments may be a matter of years, or even decades (Evans *et al.*, 1995). Dibutyltin (DBT) is the main degradation product of TBT (Mercier *et al.*, 1996) and consequently is the derivative that dominates in seawater (Mercier *et al.*, 1998). TBT degradation to DBT, thought to take place by microbial activity (Seligman *et al.*, 1988) occurs within days, although degradation may be more rapid in the warm waters of the tropics and subtropics (Mercier *et al.*, 1996).

TBT is almost universally toxic (Evans *et al.*, 1995), exerting chronic effects on aquatic organisms at concentrations down to $\leq 10\text{--}20$ ng/l (Cardwell *et al.*, 1999). A range of toxic effects have been noted in different organisms exposed to TBT including reproductive disruption (by imposex induction) in molluscs (Stewart and Thompson, 1994) and, growth rate alterations in both algae and molluscs (Beaumont and Newman, 1986; Stebbing, 1985). No known genetic loci are associated with TBT toxicity and the exact mechanisms of toxicity are unknown. A number of mechanisms have however been suggested and these

include membrane perturbation and loss of intracellular ion homeostasis (Kergosein and Rice, 1998). Both di- and trialkyltin compounds are inhibitors of oxygen uptake into tissues and the mitochondria of cells, and are potent inhibitors of ATP synthesis (Fent, 1996).

5.2 Materials and methods

Experiments were designed to assess the responses of *A. pallida* to a range of contaminants. Assessment of the following parameters of response was envisaged: lysosomal stability, levels of NOS activity and morphological status (i.e. degree of contraction/expansion). A separate set of experiments utilised arginine analogues and an NO donor to investigate the role played by NO in *A. pallida* and to explore the possible link between NO and morphological status.

Groups of *A. pallida* were transferred into glass dishes containing 70 ml seawater at least 24 hours prior to experimentation. Experimental and control anemones were maintained on a 12 h light/ 12 h dark photoperiod at 25°C. Experimental vessels were spiked with contaminants to yield expected concentration ranges of each tested contaminant that were generally environmentally realistic and/or were in line with concentrations used in previously published work with marine organisms. Anemones were additionally dosed with concentrations of copper and TBTO above environmentally realistic levels in order to assess the acute toxic response of anemones. Control anemones were incubated in LNSW, injected with an appropriate volume of LNSW, or treated with the solvent carrier as appropriate.

Material resulting from each of the approaches to lysosomal preparation (detailed in section 2.5) was examined on microscope slides before and after addition of NR. Despite application of a number of modifications of previously detailed techniques, no clearly defined lysosomal structures were visualised in any preparation thus quantification of lysosomal stability by the NR assay was not feasible. Prior to, and during contaminant

exposure total anemone diameter and, in some cases oral disc diameter were measured with callipers as detailed in section 2.7. In addition to measuring total and oral disc diameters, anemone status was visually monitored in control and experimental anemones. Anemones were noted to be attached or detached from the experimental dishes. Other specific responses, such as inflation of the pedal disc or tentacular flaccidity were noted. Anemones were considered to be dead when unresponsive to tactile stimulation. Following exposure to contaminants for specific periods (24, 48 or 72 hours), anemones were homogenised and rates of citrulline formation in host cytosolic fractions were determined by the citrulline assay (section 2.2). The Bradford assay (detailed in section 2.6) was used to determine the protein content of cytosolic aliquots.

Copper

Copper sulphate stock (10 mg/l^{-1}) was made up in LNSW. Preliminary experiments assessed anemone responses to doses of 1 and $4 \text{ } \mu\text{g/l}$ copper (as detailed in Morrall *et al.*, 1998). Subsequent pilot studies (nine separate experiments) assessed anemone responses to exposure to a greater range of copper doses (between 1 and $100 \text{ } \mu\text{g/l}$). In order to preserve stocks, single anemones were exposed to each treatment condition. Physiological responses were monitored during exposures and NOS activity was determined at the end of the incubation period (24 or 36 hours). Results from pilot studies formed the basis of a further experiment in which multiple anemones (five individuals per concentration) were dosed and incubated with between 1 and $100 \text{ } \mu\text{g/l}$ copper for 48 hours. In order to assess acute toxicity responses, additional groups of anemones were dosed and incubated with 1000 or 10,000 $\mu\text{g/l}$ copper for 24 hours. Total and oral disc diameters were recorded at intervals during this experiment. Control anemones were maintained in LNSW throughout the experimental period. All dishes of anemones (control and dosed) were gently agitated throughout the

experimental period. Groups of anemones were homogenised at the end of the incubation period and the citrulline assay was run on host cytosolic preparations.

Fluoranthene

Stocks of fluoranthene (125 or 1250 $\mu\text{g/l}$) were made up in pure acetone (reagent grade) and stored at 4°C in darkness (to prevent photooxidation) according to previously documented methods (Grundy *et al.*, 1996; Forbes *et al.*, 1996; Lowe *et al.*, 1995b). New stocks were made up each 20 days. Five separate fluoranthene dosing experiments were conducted using groups of two or three anemones per concentration. Fluoranthene was added to experimental dishes just below the water surface to give expected final concentrations of between 25 and 1000 $\mu\text{g/l}$. Solvent controls were set-up with anemones incubated with an appropriate volume of acetone (100-350 μl added to a total volume of 70 ml). In experiments lasting beyond 24 hours, LNSW was changed in all experimental dishes after 24 hours; contaminants in the solvent carrier, or solvent carrier alone was then added as appropriate. Fluoranthene dosed and control anemones were gently agitated for the duration of the experiment. Anemones were monitored at intervals during the incubation. Total anemone diameters were measured after dosing with 100-400 $\mu\text{g/l}$ fluoranthene. Groups of anemones were homogenised after either 24 or 72 hours after dose and the citrulline assay performed on the resulting preparations.

Tributyltin

A preliminary study of the responses of anemones resulting from exposure to TBTO was performed. Stocks of 100 mg/ml bis- (tri-n-butyltin) oxide (TBTO) were made up in acetone (reagent grade). Groups, each of five anemones, were dosed and incubated with concentrations between 0.1-100 $\mu\text{g/l}$ TBTO for 48 hours. In order to assess acute toxic

responses, further anemones (five anemones per group) were dosed and incubated with 1000-10 000 $\mu\text{g/l}$ for 24 hours. Solvent control groups of anemones were dosed with 10 μl acetone (equivalent to the volume added to dosed anemones). Total diameters of TBTO dosed and control anemones were measured at intervals throughout the experiment. Groups of anemones were homogenised 24 or 48 hours incubation and the citrulline assay performed.

Arginine analogue and NO donor experiments

A range of studies have used NO donors and/or NOS inhibitors to investigate the role of NO in invertebrates using whole organisms or specific tissues (Colasanti *et al.*, 1995; Colasanti *et al.*, 1997; Elphick and Melarange, 1998). Protocols from these studies were modified for use with *A. pallida*. The arginine analogues HCl (L-NAME) and N^o-Nitro-L-arginine (L-NNA) or the NO donor sodium nitroprusside (SNP) was administered to whole live anemones and the morphological responses of the organisms were noted. The citrulline assay was performed on anemones subjected to most treatments. The mechanism of NO release from sodium nitroprusside is incompletely understood but it is known that exposure to light or a one-electron reduction is required (Feelisch and Stamler, 1997a). In biological systems, both enzymatic and non-enzymatic NO release from SNP may occur (Feelisch and Stamler, 1997a).

Solutions of L-NNA and L-NAME were made up in LNSW. SNP was made up in deionised water. Inhibitor or donor solutions were well mixed with LNSW in experimental dishes of anemones at the start of the incubation period. Alternatively, anemones were injected with inhibitor solutions into the pharynx via the mouth with a flat tipped Hamilton syringe. Treatment concentrations, mode of administration and exposure periods are outlined in table 5.1.

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Treatment dose	Mode of exposure	Incubation period (hours)
50 μ M L-NAME or L-NNA	incubation	3
100 μ M L-NAME or L-NNA	incubation	3
50 μ M L-NAME or L-NNA	injection	3
100 μ M L-NAME or L-NNA	injection	3
25 μ M L-NAME or L-NNA	injection	48
50 μ M L-NAME or L-NNA	injection	48
50 μ M L-arginine	injection	48
100 μ M L-arginine	injection	48
25 μ M L-NAME or L-NNA + 50 μ M L-arg	injection	48
50 μ M L-NAME or L-NNA + 100 μ M L-arginine	injection	48
20 μ M SNP	incubation	24
100 μ M SNP	incubation	24

Table 5.1. Outline of concentration, mode of administration and exposure period of whole anemones treated with arginine analogues or the NO donor SNP.

Anemones incubated in 1 or 5 mM L-NAME and L-NNA or LNSW alone (controls) were photographed immediately prior to addition of analogues, then each hour up until the end of the three-hour incubation period.

5.3 Results

Neutral red

No structures resembling lysosomes were visualised in any of the preparations of *A. pallida*.

Within the time constraints of this research, assessment of lysosomal stability was deemed to be impractical for use as an indicator of the response of *A. pallida* to contaminants.

Copper exposures

Pilot studies showed 24-hour exposures to copper doses of between 1-100 µg/l to be sublethal to *A. pallida*. Detachment of anemones from experimental dishes was frequently observed in both control and copper exposed anemones. Size reductions, (due to contraction of tentacles and/or body columns) were noted in anemones incubated in LNSW both with and without added copper; contractions were generally most pronounced in anemones exposed to the highest concentration tested in each experiment (e.g. table 5.2).

Treatment (µg/l copper)	Start and end size (mm)	Maximum size change during exposure period (mm)	% size change pre dose- end 24 hour incubation
Control	30.0-15.0	15.0	-50
25 µg/l	31.0-6.0	25.0	-81
50 µg/l	28.0-6.0	22.0	-79
75 µg/l	10.0-3.5	6.5	-65
100 µg/l	26.0-3.5	22.5	-87

Table 5.2. Size response of anemones exposed to copper concentrations between 25-100 µg/l. Measurements of individual anemones were made immediately prior to dose (start size), then at intervals up to 24 hours after dose. End size was determined at the end of experimental exposure period. Maximum size change represents the difference between the minimum and maximum size observed over the experimental period. The percent size change was calculated from pre incubation to post 24-hour incubation size. Values shown are from a single anemone per treatment.

Size changes noted in control anemones were typically not consistent through experimental periods and tended to be less extreme than changes noted in anemones exposed to copper.

Pilot studies showed NOS activity levels in anemones exposed to copper to be different from control values but much variability was evident within and between experiments. For example, *A. pallida* dosed with 50 µg/l copper exhibited NOS activity levels that were 87% higher than control values, while anemones dosed with 100 µg/l exhibited NOS activity levels 41% lower than control animals. No clear dose response between NOS activity and copper concentration was established by use of single anemones, but the sublethal nature of short-term exposure to copper doses between 1-100 µg/l was established and details on morphological responses to this concentration range were obtained. These results were used as the basis for further studies using multiple anemones per treatment.

No clear dose response relationship was shown between NOS activity and copper concentration when groups of *A. pallida* were exposed to 0.1-100 µg/l copper for 48 hours (figure 5.1). NOS activity levels in anemones exposed to 0.1-10 µg/l for 48 hours were significantly different from controls ($p < 0.05$); both increased and reduced activity levels were detected in *A. pallida* exposed to 0.1-100 µg/l copper for 48 hours. The biggest alteration in enzyme activity was detected in anemones exposed to 0.1 µg/l copper in which NOS activity levels were 80% higher than in controls. NOS activity levels in anemones exposed to 100 µg/l for 48 hours were not statistically significantly different from controls ($p < 0.05$). Anemones exposed to 1000 µg/l for 24 hours had levels of NOS activity that were elevated (by 37%) and significantly different from controls ($p < 0.05$) (figure 5.2).

No mortality occurred in anemones incubated in between 0.1 and 100 µg/l copper for 48 hours, or 1000 µg/l copper for 24 hours. Anemones incubated in 10,000 µg/l copper were

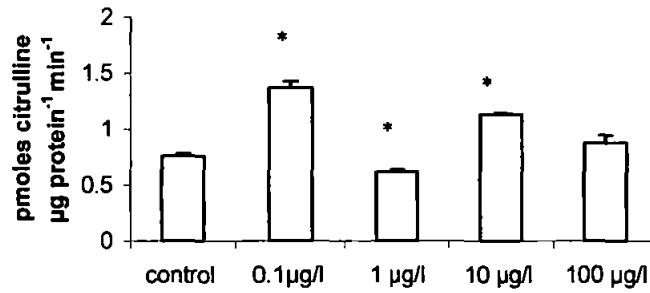


Figure 5.1. Rates of citrulline formation in anemones subject to 48-hour incubation with copper concentrations between 0.1-100 $\mu\text{g/l}$. Values shown are means of triplicate determinations using cytosol from pooled homogenate of five anemones subject to each treatment. Asterisks denote values statistically significant different from controls, error bars show standard deviations.

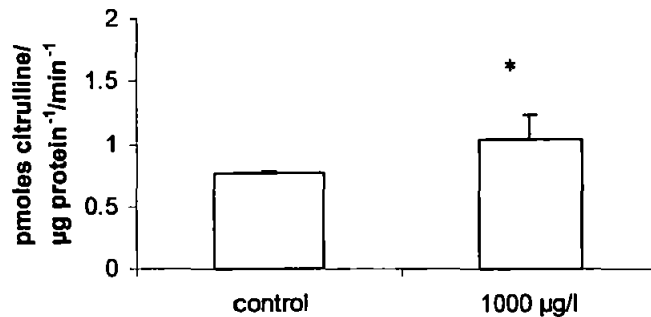


Figure 5.2. Rates of citrulline formation in anemones subject to 24-hour incubation with copper concentrations of 1000 $\mu\text{g/l}$. Values shown are means of triplicate determinations using cytosol from pooled homogenate of five anemones subject to each treatment. Asterisks denote values statistically significant different from controls, error bars show standard deviations.

surrounded by a white mucous layer and were unresponsive to tactile stimulation after 24 hours; these animals were considered to be dead and were not assayed for NOS activity. Both control anemones and those incubated in copper concentrations between 0.1 and 100 $\mu\text{g/l}$ for 48 hours reduced in terms of their total diameter (table 5.3). Size changes were due to changes in the expansion or contraction of tentacles, or withdrawal of tentacles. No clear relationship between copper concentrations and morphological status was evident in anemones exposed to 0.1-100 $\mu\text{g/l}$ copper, but anemones incubated in 100 $\mu\text{g/l}$ exhibited the most extreme size reduction (69% from pre-dose to end of incubation) (table 5.3).

Treatment ($\mu\text{g/l}$ copper)	Start and end size (mm)	% size change pre dose-end of incubation period
Control	15.5-8.5	-45
0.1 $\mu\text{g/l}$	13.9-6.0	-57
1 $\mu\text{g/l}$	13.6-11.8	-13
10 $\mu\text{g/l}$	17.5-11.4	-35
100 $\mu\text{g/l}$	18.4-9.1	-51

Table 5.3. Total diameter of *A. pallida* incubated in LNSW (control) or copper at concentrations between 0.1 and 100 $\mu\text{g/l}$ over a 48-hour period. Measurements of individual anemones were made immediately prior to dose (start size), then at intervals up to 48 hours after dose. End size was determined at the end of experimental exposure period. The percent size change was calculated from pre incubation to post 48-hour incubation size. Values shown are means of five anemones per treatment.

Control anemones incubated for 24 hours in LNSW reduced in size by 44%, while those incubated in 1000 or 10,000 $\mu\text{g/l}$ copper for the same incubation period exhibited more extreme size reductions (88 and 80% respectively) (table 5.4). The majority of the size reduction seen in anemones incubated in 1000 or 10,000 $\mu\text{g/l}$ copper occurred within 30 minutes of addition of copper (76 and 77% of overall size reduction respectively). All animals exposed to 1000 or 10,000 $\mu\text{g/l}$ were detached from the dish after 4 hours incubation and re-attachment was not observed within the experimental period. Half (five) the control anemones became detached during 24 hours incubation. Expelled zooxanthellae

were visible in the dish surrounding anemones incubated in 1000 or 10,000 µg/l copper; the tentacle tips of anemones incubated in 1000 µg/l copper were observed to have ruptured at the end of the incubation period.

Treatment (µg/l copper)	Start and end size (mm)	% size change pre dose-end of incubation period
Control	15.5-15.1	-3
1000 µg/l	25.6-3.1	-88
10,000 µg/l	23-0	-

Table 5.4. Total diameter of *A. pallida* incubated in LNSW (control) or copper at concentrations of 1000 or 100,00 µg/l over a 24-hour period. Measurements of individual anemones were made immediately prior to dose (start size), then at intervals up to 48 hours after dose. End size was determined at the end of experimental exposure period. The percent size change was calculated from pre incubation to post 24-hour incubation size. Values shown are means of five anemones per treatment. Anemones incubated in 100,00 µg/l were dead after 24 hours.

Fluoranthene exposures

Levels of NOS activity in anemones dosed with 25-1000 µg/l fluoranthene for 24-72 hours were similar to levels detected in LNSW or solvent controls. Results from three separate fluoranthene exposure experiments (concentrations 25-100 µg/l) are shown in tables 5.5 A, B and C. These results are representative of those obtained from all of the fluoranthene exposure experiments conducted. Inter-assay variance (discussed in detail in Chapter 3) accounts for the differences in enzyme activity between experimental data sets.

Anemones exposed to fluoranthene between 25-1000 µg/l or the solvent carrier alone exhibited a range of morphological responses including alterations in size and detachment from experimental dishes. Both control anemones and those exposed to 100-400 µg/l fluoranthene reduced in size from start to end of the experimental period; the most marked reduction was observed in anemones exposed to 300 or 400 µg/l (with size reductions of 64 and 63% noted respectively) (table 5.6). Anemones incubated with the solvent carrier alone

(acetone) increased in size (by 67%) during the incubation period. Anemones exposed to fluoranthene or the solvent carrier alone frequently detached from the experimental vessel and enlarged column bases were often noted.

A Treatment	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ \pm s.d.
Control	2.7 \pm 0.04
Solvent control	2.6 \pm 0.05
25 $\mu\text{g/l}$	2.6 \pm 0.07
50 $\mu\text{g/l}$	2.6 \pm 0.05
75 $\mu\text{g/l}$	2.6 \pm 0.03
100 $\mu\text{g/l}$	2.6 \pm 0.07

B Treatment	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ \pm s.d.
Control	1.8 \pm 0.07
Solvent control	1.7 \pm 0.02
100 $\mu\text{g/l}$	1.8 \pm 0.04
200 $\mu\text{g/l}$	1.9 \pm 0.06
300 $\mu\text{g/l}$	1.9 \pm 0.03
400 $\mu\text{g/l}$	1.9 \pm 0.02

C Treatment	Pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$ \pm s.d.
Control	2.5 \pm 0.04
Solvent control	2.4 \pm 0.05
250 $\mu\text{g/l}$	2.1 \pm 0.02
500 $\mu\text{g/l}$	2.3 \pm 0.06
750 $\mu\text{g/l}$	2.3 \pm 0.03
1000 $\mu\text{g/l}$	2.4 \pm 0.01

Table 5.5 A, B and C. Rates of citrulline formation in anemones subject to 24 hour incubation with fluoranthene concentrations between 25-100 $\mu\text{g/l}$ (A), 100-400 $\mu\text{g/l}$ (B) and 250-1000 $\mu\text{g/l}$ (C). Controls were maintained in LNSW for the experimental period; solvent control anemones were incubated with acetone concentrations equivalent to that used in the fluoranthene dose. Values shown are means of triplicate determinations using cytosol from pooled homogenate from two anemones subject to each treatment.

Treatment ($\mu\text{g/l}$ fluoranthene)	Start and end size (mm)	% size change pre dose-end of incubation period
Control	13.0-8.8	-33
Acetone control	6.0-10.0	+67
100 $\mu\text{g/l}$	16.5-13.0	-21
200 $\mu\text{g/l}$	18.0-13.0	-28
300 $\mu\text{g/l}$	14.0-5.0	-64
400 $\mu\text{g/l}$	12.0-4.5	-63

Table 5.6. Size response of anemones exposed to fluoranthene concentrations of 100-400 $\mu\text{g/l}$. Measurements of individual anemones were made immediately prior to dose (start size), then at intervals up to 24 hours after dose. End size was determined at the end of experimental exposure period. The percent size change was calculated from pre incubation to post 24-hour incubation size. Values shown are means of two anemones per treatment.

Anemones maintained in LNSW remained attached to the experimental vessels. No clear relationship between anemone morphological responses and NOS activity was shown in the results of *A. pallida* exposed to fluoranthene.

TBTO exposures

No statistically significant difference ($p < 0.05$) was detected in NOS activity levels of anemones incubated in TBTO concentrations between 0.1 and 100 $\mu\text{g/l}$ for 48 hours and either the LNSW or solvent controls; NOS activity levels ranged between 5.38 ± 0.01 and 6.44 ± 0.37 (means and s.d. of triplicate determinations) with an overall mean of 5.76 ± 0.65 ($n=6$; each sample consisted of cytosol prepared from five individuals). NOS activity levels in anemones exposed to both 1000 and 10,000 $\mu\text{g/l}$ TBTO were statistically significantly different from the LNSW controls; 38 and 107% increases in activity were shown respectively relative to LNSW controls. Expelled zooxanthellae were noted in dishes containing anemones exposed to 1000 and 10,000 $\mu\text{g/l}$ TBTO. Increased mucus production was observed on the surface of anemones exposed to 10,000 $\mu\text{g/l}$, no other experimental anemones exhibited this.

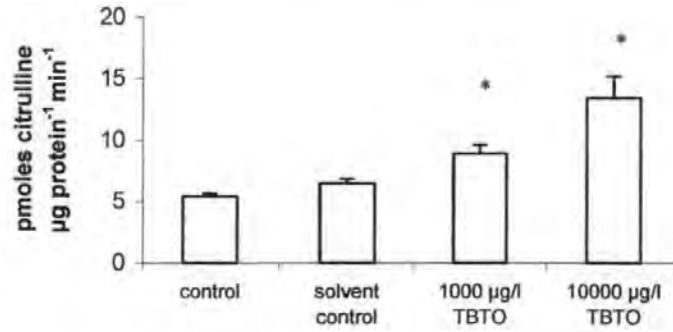


Figure 5.3. Rates of citrulline formation in control anemones and those incubated in 1000 or 10,000 $\mu\text{g/l}$ TBTO. Control anemones were incubated in LNSW for the duration of the experiment; solvent control anemones were incubated in acetone at a concentration equivalent to that applied to the TBTO exposed anemones. Values shown are means of triplicate determinations using cytosol from pooled homogenate from five anemones subject to each treatment. Asterisks denote values statistically significantly different from controls; error bars show standard deviations.

While the protein contents of cytosolic preparations of anemones incubated in 0.1-100 $\mu\text{g/l}$ TBTO for 48 hours were little changed from LNSW control and solvent controls, anemones exposed to ≥ 1000 $\mu\text{g/l}$ TBTO for 24 hours had reduced protein contents relative to LNSW and solvent control anemones (figure 5.4). Protein contents were reduced by 49 or 63% in anemones dosed with 1000 or 10,000 $\mu\text{g/l}$ TBTO respectively relative to LNSW controls. Anemones incubated in LNSW alone, or in 0.1-100 $\mu\text{g/l}$ TBTO reduced in size from pre dose to the end of 48 hours incubation; solvent carrier control anemones showed a slight increase in size over 48 hours (1%) (table 5.7).

48 hour incubation	Start-end size (mm)	Maximum size change during exposure period (mm)	% size change pre dose-end of incubation period
Control	25.0-16.0	13.1	-36
Solvent control	18.0-18.2	9.3	+1
0.1 $\mu\text{g/l}$ TBTO	27.6-23.3	12.2	-16
1 $\mu\text{g/l}$ TBTO	24.8-10.1	18.0	-60
10 $\mu\text{g/l}$ TBTO	25.3-12.4	15.9	-52
100 $\mu\text{g/l}$ TBTO	25.8-10.4	17.4	-60

Table 5.7. Size response of anemones exposed to TBTO concentrations between 0.1-100 $\mu\text{g/l}$. Measurements (total diameter) were made immediately prior to dose (start size), then at intervals up to 48 hours after dose. End size was determined at the end of experimental exposure period. Maximum size change represents the difference between the minimum and maximum size observed over the experimental period. The percent size change was calculated from pre incubation to post 48-hour incubation size. Values shown are means of five anemones per treatment.

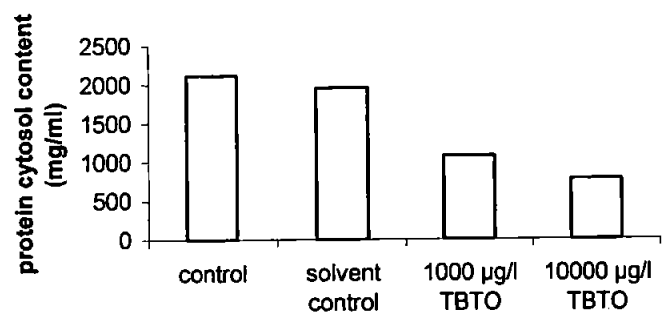


Figure 5.4. Protein contents of cytosolic preparations of control anemones (incubated in LNSW), solvent controls (LNSW and acetone) and anemones incubated in 1000 or 10, 000 µg/l TBTO for 24 hours. Values shown were determined using cytosol from pooled homogenate from five anemones from each treatment.

Anemones incubated in LNSW or in solutions of 1000 or 10,000 $\mu\text{g/l}$ TBTO reduced in size over 24 hours, (table 5.8). Anemones incubated in 1000 or 10,000 $\mu\text{g/l}$ TBTO contracted immediately upon addition of TBTO and those incubated in 10,000 $\mu\text{g/l}$ were dead after 24 hours incubation. Size reductions observed in anemones incubated in 1000 or 10,000 $\mu\text{g/l}$ for 24 hours were greater (74 and 79% respectively) than those observed in anemones incubated in 0.1-100 $\mu\text{g/l}$ for 48 hours (maximum reduction 59.7 shown in response to 1 $\mu\text{g/l}$ and 100 $\mu\text{g/l}$ TBTO). Anemones incubated with acetone alone for 24 hours increased in size (by 17%).

24 hour incubation	Start-end size (mm)	Maximum size change during exposure period (mm)	% size change pre dose-end of incubation period
Control	28.0-16.0	12.0	-43
Solvent control	7.8-9.2	6.4	+17
1000 $\mu\text{g/l}$ TBTO	21.6-5.7	17.1	-74
10,000 $\mu\text{g/l}$ TBTO	22.3-4.6	17.7	-80

Table 5.8. Size response of anemones exposed to TBTO concentrations of 1000 or 10,000 $\mu\text{g/l}$. Total diameter measurements were made immediately prior to dose (start size), then at intervals up to 24 hours after dose. End size was determined at the end of experimental exposure period. Maximum size change represents the difference between the minimum and maximum size observed over the experimental period. The percent size change was calculated from pre incubation to post 24-hour incubation size. Values shown are means of five anemones per treatment.

No clear relationship between anemone morphological responses and NOS activity was shown in the results of TBTO exposure experiments.

Responses of whole anemones to exposure to arginine analogues

Whole anemones immersed in solutions of 1 or 5 mM of the arginine analogues L-NAME or L-NNA were observed generally to reduce in size relative to appropriate controls over a three-hour incubation period (table 5.9 and figure 5.5C and 5.6E and 5.6D and 5.6F).

Three hour incubation	Start and end size mm (maximum size range (mm) exhibited over entire period)		% size change pre dose-end of incubation period	
Treatment	total diameter	oral disc diameter	Total diameter	oral disc diameter
Control	13.0-11.8 (1.2)	4.3-4.7 (0.9)	-9	+9
1 mm L-NAME	15.3-6.0 (9.3)	4.3-3.0 (1.5)	-61	-30
1 mm L-NNA	10.3-8.2 (2.2)	4.2-2.0 (2.2)	-20	-52
Control	11.0-10.5 (1.3)	3.8-3.6 (1.3)	-5	-5
5 mM L-NAME	13.0-7.7 (6.0)	3.7-2.2 (1.5)	-41	-41
5 mM L-NNA	10.3-3.0 (7.3)	4.0-1.0 (3.0)	-67	-75

Table 5.9. Total and oral disc diameters of anemones incubated for 3 hours in 1 or 5 mM L-NAME or L-NNA, or in LNSW (control). Measurements were made immediately prior to analogue addition (start size) and at intervals over the incubation period. End size was determined at the end of experimental exposure period. Maximum size change (values in parentheses in columns 2 and 3) represents the difference between the minimum and maximum size observed over the experimental period. The percent size change is calculated from pre incubation to post 3-hour incubation size. Values shown are means of 3 anemones per treatment.

Size reductions were due to both tentacle and column contraction. Control anemones (incubated in LNSW) showed little change in size over the experimental period (table 5.9 and figures 5.5A and 5.6B). The most extreme size reduction was observed in anemones incubated in 5 mM L-NNA; anemones subject to this treatment detached from the experimental vessel and were observed to be lying on their sides with very contracted tentacles and columns (figure 5.6F). Zooxanthellae were observed in the dish containing anemones incubated in 5 mM L-NNA.

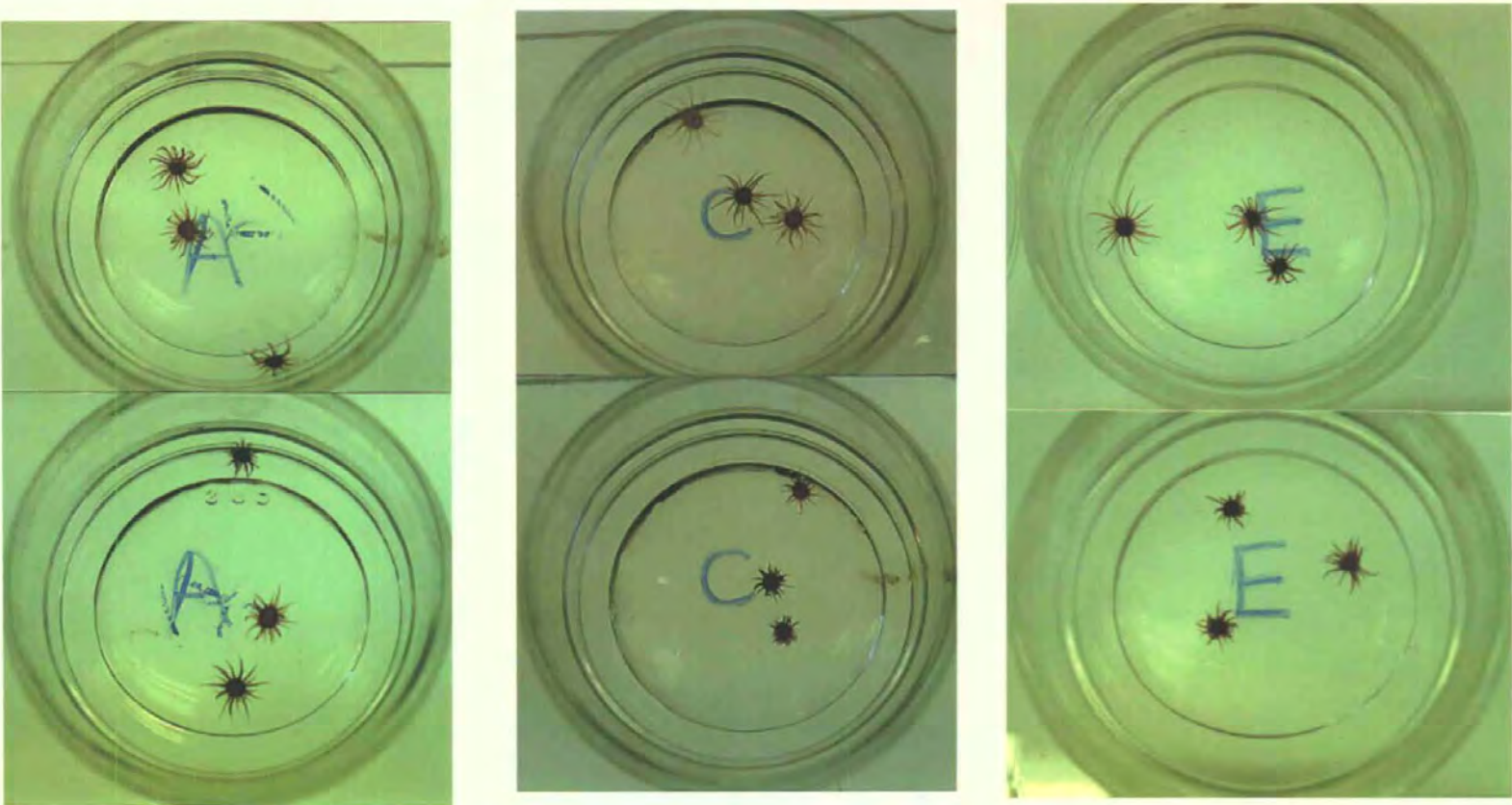


Figure 5.5 A, C and E. Anemones incubated in LNSW (A), 1 mM L-NAME (C) and 1 mM L-NNA (E). Upper images (I) illustrate pre-dose condition, lower images (II) illustrate anemones 3 hours after dose. Scale bar (lower right) represents approximately 2 cm.

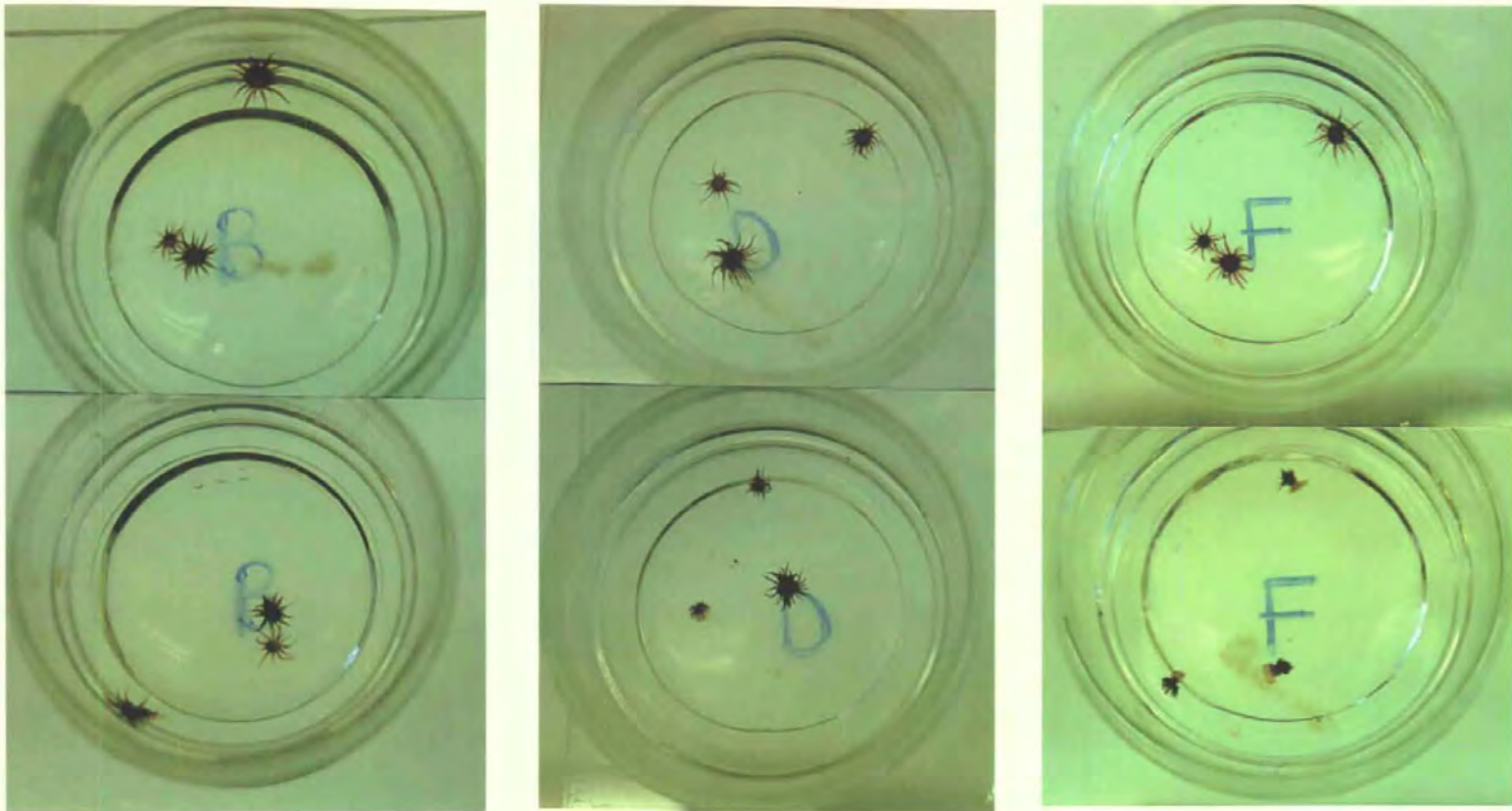


Figure 5.6 B, D and F. Anemone injected with LNSW (B), 5 mM L-NAME (D) or 5 mM L-NNA. Upper images (I) illustrate pre-dose condition, lower images (II) illustrate anemones 3 hours after dose. Arrows point to extremely contracted anemones. Scale bar (lower right) represents approximately 2 cm.

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Injection of 50 or 100 μM L-NAME into anemones resulted in little morphological change over three hours following injection while those injected with the same concentrations of L-NNA reduced in size over the same period (table 5.10). Anemones injected with LNSW volumes equivalent to 50 μM treatments showed a slight decrease in size (-7%), while the controls for 100 μM increased in size over the incubation period (+18%).

Three hour incubation post injection	Start and end size (mm)	Maximum size range exhibited over entire period (mm)	% size change pre dose-3 hour incubation period
Control	15.0-14.0	3.7	-7
50 μM L-NAME	26.7-27.7	10.3	+4
50 μM L-NNA	23.3-13.7	14.0	-41
Control	16.3-19.3	4.3	+18
100 μM L-NAME	24.3-23.3	2.6	-4
100 μM L-NNA	30.7-13.7	20.0	-55

Table 5.10. Size responses of *A. pallida* injected with L-NAME, L-NNA or equivalent volumes of LNSW (controls) then incubated for 3 hours. Total diameter measurements were made immediately prior to injection of analogues (start size), at intervals over the following incubation period. End size was determined at the end of experimental exposure period. Maximum size change represents the difference between the minimum and maximum size observed over the experimental period. The percent size change is calculated from pre incubation to end of incubation size. Values shown are means of three anemones per treatment.

Pilot studies of the responses of *A. pallida* to longer term incubations (48 hours) following injection of 25 or 50 μM L-NAME or L-NNA showed reduction of anemone size to be a consistent response to these inhibitors. Control anemones injected with LNSW showed either no change in size or slight increases in size. Co-injection of arginine analogues and L-arginine (25 or 50 μM L-NAME or L-NNA with 50 or 100 μM L-arginine respectively) followed by a 48-hour incubation period generally increased the contraction response

exhibited by injection of the arginine analogues alone. Injection of 50 or 100 μM L-arginine alone followed by a 48-hour incubation period resulted in dramatic reduction of size.

NOS activity data for whole anemones treated with arginine analogues are presented in chapter three. No clear relationship between anemone morphological responses and NOS activity was shown in the results of arginine analogue treatments.

NO donor exposures

Control anemones incubated in LNSW over 24 hours showed increases in both total and oral disc diameter (table 5.11). Both total and oral disc diameters of whole anemones incubated in 20 μM SNP reduced during 24 hours of exposure. Anemones incubated in solutions of 100 μM SNP showed little size change in terms of their oral disc diameters over 24 hours but their total diameters were increased slightly more than control anemones (26 % relative to 20%).

24 hour incubation	Start and end size (mm)		% size change pre dose-end of incubation period	
Treatment	total diameter	oral disc diameter	total diameter	oral disc diameter
Control	14.3-17.1	4.7-5.7	+20	+21
20 μM SNP	22.8-13.7	5.7-4.9	-40	-14
100 μM SNP	17.4-21.9	5.0-5.1	+26	+2

Table 5.11. Size responses of *A. pallida* incubated in 20 or 100 μM SNP for 24 hours. Total diameter measurements were made immediately prior to injection of analogues (start size), at intervals over the following incubation period. End size was determined at the end of experimental exposure period. Percent size changes were calculated from start and end sizes. Values shown are means of six anemones per treatment.

NOS activity data for whole anemones treated with SNP are presented in chapter three. No clear relationship between anemone morphological responses and NOS activity was shown in the results of whole anemones treated with SNP.

5.4 Discussion

Neutral red assay outcome

Visualisation of lysosomes from *A. pallida* was not achieved in this study. As a result, interpretation of NOS activity levels in contaminant-exposed anemones was done without cross-reference to an assessment of cellular stress. The reason for the failure to prepare and visualise lysosomal structures from *A. pallida* are unknown at this stage. It is possible that mucus produced during preparation of *A. pallida* prevented successful lysosomal isolation. Alternatively, *A. pallida* lysosomal ultrastructure may be incompatible with the assay. The amorphous nature of the anemones tissue and/or possibly an uneven distribution of lysosomes within the anemones tissues may also have contributed to the lack of success of this assay. Further work is needed to develop techniques for the preparation of cnidarian lysosomes that will enable use of the NR assay with species from this phylum.

Contaminant concentrations

Contaminant doses used in the studies documented here were nominal and actual concentrations of experimental vessels were not analysed. This was a serious oversight of experimental design that has profound implications for all the results reported here. As it is not feasible to quantify the actual contaminant concentrations which experimental anemones were exposed to retroactively, the results are reported here with the concentrations expected from addition of known volumes of stock contaminants to experimental vessels. Prior to future studies of contaminant responses of anemones, the bioavailability of the chemicals to be tested should be determined.

Effects elicited by *A. pallida* exposed to copper

While variations in NOS activity were shown in preparations of anemones exposed to copper, no pattern of dose response in NOS activity levels was evident in the concentration range and exposure periods tested. The biological significance of the non-dose dependent alterations in NOS activity levels observed in anemones exposed to concentrations of copper between 0.1 and 100 $\mu\text{g/l}$ is not clear, but the observed deviations in activity levels from control values suggest that enzyme activity was affected by copper. Exposure to copper may have affected NOS activity by interacting with the enzyme substrate or cofactors, or by influencing the physical characteristics of the intracellular environment. Further work is needed to establish the reason for the observed changes in levels of enzyme activity and to elucidate the biological significance of these changes (in particular with regard to changes in rates of NO formation). These findings provide a basis for further studies but do not themselves provide evidence of involvement of NOS in the short-term toxic response of *A. pallida* to copper.

Exposure of *A. pallida* to copper concentrations $\leq 100 \mu\text{g/l}$ for 48 hours or 1000 $\mu\text{g/l}$ for 24 hours was sublethal; however, mortality resulted from exposure to 10,000 $\mu\text{g/l}$ copper for 24 hours. Previous work has shown copper concentrations of $\geq 100 \mu\text{g/l}$ to be sufficient to kill the coral species *Pocillopora damicornis* and *Montopora verrucosa* within 24 hours (Evans, 1977). These results suggest that *A. pallida* is more tolerant of copper than these coral species.

A. pallida responded to all copper concentrations tested by contracting. Although control anemone sizes were not static for the experimental duration, the size reductions shown by anemones exposed to copper generally exceeded those shown by control animals. It is possible that part of the contraction exhibited by all experimental anemones was due to the agitation applied. While a strict dose response relationship between degree of contraction

and copper concentration was not evident, the highest concentration tested in each experiment consistently resulted in the greatest size reduction. In addition, rapid size reductions (within 30 minutes of dose) were observed in anemones exposed to 1000 or 10,000 µg/l. Exposure of the coral species *Pocillopora damicornis* and *Montopora verrucosa* to 10 µg/l copper sulphate was reported to result in polyp withdrawal (Evans, 1977), a response equivalent to the tentacle withdrawal that accounted for the majority of the size reduction shown by *A. pallida* in response to exposure to copper. The significance and implications of the degree of contraction in anemones is discussed later in this section.

As detachment from experimental dishes was observed in both control and copper exposed anemones, it is possible that this response, along with at least some of the contraction responses exhibited, resulted from the agitation applied to the experimental dishes to maintain the homogeneity of applied treatments. Detachment has been previously noted in anemones exposed to adverse stimuli (Edmunds *et al.*, 1976).

Exposure to high concentrations of copper (1000 and 10,000 µg/l) resulted in disturbance of the symbiotic relationship between host and zooxanthellae in *A. pallida* within 24 hours as shown by the presence of zooxanthellae disassociated from the anemones in the experimental dishes. A wide range of adverse environmental conditions have been noted to result in zooxanthellae loss in organisms that harbour these symbionts (detailed previously in section 1.7). Zooxanthellae loss has a number of implications for the symbiotic assemblage and is generally thought to be detrimental to both host and symbiont. The marked ability of algae to accumulate copper has been previously documented (Denton and Burdon-Jones, 1986) and zooxanthellae have been implicated in the uptake, regulation and excretion of metals by symbiotic cnidarians (Harland and Nganro, 1990; Harland *et al.*, 1990) and clams (Dusquesne and Coll, 1995). Further studies are required to investigate the

role played by the zooxanthellae associated with *A. pallida* in the uptake, accumulation and elimination of copper.

A thicker than normal layer of mucus was observed on the surfaces of anemones exposed to 10,000 µg/l copper, suggesting increased mucus production, a response that has previously been noted in cnidarians exposed to heavy metals (Thompson *et al.*, 1980; Harland and Nganro, 1990; Mercier *et al.*, 1998). It has been suggested that mucus plays a role in the regulation of copper concentrations of cnidarians by provision of sites for bindings or chelation of the metal ion (Harland and Nganro, 1990). In addition, it is likely that increased mucus production would provide a degree of protection from water borne contaminants by increasing the diffusion distance, thus potentially reducing contaminant uptake.

Effects elicited by *A. pallida* exposed to fluoranthene

NOS activity levels were unchanged by exposure of whole anemones to 25-1000 µg/l fluoranthene for 24-72 hours. This indicates that interactions between the contaminant and NOS related biochemistry were absent or insufficient to affect the enzyme activity. An absence of toxicity of aqueous solutions of fluoranthene has previously been reported in *Mytilus edulis* subject to 72 or 96-hour exposures and has been attributed to the exposure period that may have been insufficient to permit bioconcentration of a toxic body burden (Donkin *et al.*, 1991). In addition, these authors noted that relatively soluble PAHs (such as fluoranthene) may crystallise within organisms or may be sequestered by some other mechanism (Donkin *et al.*, 1991) thereby reducing the biological availability of the chemical. Thus the short exposure period used in these studies and/or potential sequestration of fluoranthene may have been responsible for the lack of interaction with NOS. Alternatively, it is possible that *A. pallida* is capable of metabolising fluoranthene (as is *Capitella* sp. (Forbes *et al.*, 1996)) and as such is able to avoid interactions with biochemical components such as NOS.

The similarity of NOS activity levels in control and fluoranthene exposed anemones contrasts with the variable NOS activity levels shown in copper exposed anemones. Comparison of the observed NOS activity levels in anemones exposed to these two contaminants highlights the lack of responsiveness of *A. pallida* NOS to fluoranthene exposure and the variability in NOS activity levels that resulted from exposure to copper.

Size reductions were observed in anemones incubated in LNSW alone or with fluoranthene. Although a strict dose dependent response to fluoranthene was not shown, anemones exposed to the highest concentrations generally showed the greatest contractions. The non-specific narcotic properties of acetone have been previously described (Lowe *et al.*, 1996b) and may be at least partly responsible for the observed increased size of the carrier control. As detachment was observed in anemones exposed to fluoranthene or the solvent carrier alone but not in the LNSW controls, it is likely that the solvent elicited the response. It must be noted that detachment responses were not noted in experiments that investigated responses to TBTO despite the use of acetone as solvent carrier. This may be due to the lower volumes of solvent used in these experiments (which were at least an order of magnitude less). The agitation applied to the experimental vessels may have also contributed to the observed detachment response. The significance and implications of morphological changes are discussed later in this section.

Effects elicited in *A. pallida* exposed to TBTO

NOS activity levels were not significantly changed in *A. pallida* exposed to 0.1-100 µg/l TBTO for 48 hours suggesting that the NOS enzyme was unaffected by short term exposures to these concentrations. NOS activity levels were however elevated in anemones exposed to ≥1000 µg/l TBTO for 24 hours. The reasons for the marked increase in NOS activity levels observed in *A. pallida* exposed to very high TBTO concentrations (≥1000

µg/l) is unclear, but the expressed activity levels will have been strongly influenced by the dramatic decline in host protein contents detected in these anemones. Metabolism of TBT by marine animals is usually correlated with the presence of cytochrome P-450 dependent mixed-function oxidase system (Fish *et al.*, 1976; Lee, 1991). The P450 system is inhibited by TBT by binding to critical thiol groups at the active site; as the catalytic portion of the NOS enzyme includes a heme system similar to cytochrome P450 reductase (Kergosein and Rice, 1998), a similar inhibitory effect at the NOS active site is possible (Smith *et al.*, 2000). In light of this suggested mechanism of action, enhancement of NOS activity by high concentrations of TBT was an unexpected outcome and is not easily explained. Previous studies have reported both increased and reduced levels of NOS activity in cells and biological fluids exposed to a range of TBTO concentrations (Kergosein and Rice, 1998; Smith *et al.*, 2000). Stimulation of NO production has been noted in the response of murine macrophages and mussel haemocytes exposed to low doses of TBT (0.3 or 3.0 mM TBTO/kg and 10 µg/l TBTO respectively) (Kergosein and Rice, 1998; Smith *et al.*, 2000). The increased level of NOS activity shown in mice macrophages was attributed to a local inflammatory response and it has been suggested that increased activity represents stimulation of the stress response (Kergosein and Rice, 1998). In contrast to the stimulated NOS activity noted in murine macrophages and mussel haemolymph to low doses of TBTO, reduced activity was reported in response to higher concentrations (mice dosed with 30 mM/kg and mussel exposed to 100 µg/l) (Kergosein and Rice, 1998; Smith *et al.*, 2000). NOS activity inhibition has been proposed to occur via direct enzyme inhibition or as a secondary consequence of metabolic toxicity (Smith *et al.*, 2000).

No change from control levels was observed in the protein contents of cytosolic preparations from anemones exposed to TBTO concentrations up to 100 µg/l. These findings contrast to those of Smith *et al.*, (2000) who reported that the protein contents of

haemolymph from *M. edulis* rose slightly following exposure to TBTO up to 100 µg/l. The dramatic reduction in protein contents of cytosolic preparations of anemones exposed to 1000 or 10, 000 µg/l TBTO indicate contaminant-induced breakdown of anemone protein. These findings are the first to report that exposure to TBTO affects the protein contents of sea anemones. Stress has previously been reported to accelerate protein catabolism in man and animals (Kasschau *et al.*, 1980).

The morphological changes of *A. pallida* exposed to TBTO reported in this study are in line with the results of previous studies of the response of anemones to TBT; both volume reductions by contraction and tentacle contraction have been reported (Mercier *et al.*, 1997; Mercier *et al.*, 1996). Levels of illumination have been previously shown to influence the uptake and accumulation of butyltins from seawater and *A. pallida* maintained in light have been shown to accumulate twice the level of DBT as those maintained in the dark (Mercier *et al.*, 1996). The significance and implications of the observed changes in morphological status in TBTO exposed anemones (including alterations in incident illumination that result from these changes) are discussed later in this section.

Zooxanthellae loss was noted in *A. pallida* exposed to TBTO at concentrations ≥ 1000 µg/l for 24 hours indicating disruption of the host-zooxanthellae symbiosis. Zooxanthellae loss has been previously reported in *A. pallida* exposed to longer-term lower TBT concentrations (50 ng/l for up to 28 days) (Mercier *et al.*, 1997; Mercier *et al.*, 1996). Zooxanthellae have been reported to play a major role in the uptake and regulation of TBT from water in symbiotic anemones (Mercier *et al.*, 1996). Carbon and nutrients are made available to sea anemones by zooxanthellae mediated uptake from seawater; owing to their partly organic nature, it has been suggested that butyltins may be taken up by zooxanthellae as readily as carbon (Mercier *et al.*, 1996). Numerous species of algae are able to rapidly degrade TBT (Mercier *et al.*, 1996) and zooxanthellae have been reported to be of crucial importance in

the degradation and depuration processes observed in symbiotic cnidarians (Mercier *et al.*, 1998). Depuration of butyltins has been reported to occur in *A. pallida* within a day (Mercier *et al.*, 1998) via zooxanthellae loss. While depuration by this means will reduce contaminant burdens of the symbiosis, it nevertheless is likely to be detrimental to the symbiosis (Mercier *et al.*, 1996). Increased mucus production, observed in this study in anemones exposed to 10,000 µg/l for 24 hours, has previously been reported in a temperate anemone exposed to much lower concentrations of TBTO (50 ng/l) for the same period (Mercier *et al.*, 1998). Increased mucus production was however not reported in *A. pallida* exposed to these same TBT concentrations for up to 28 days (Mercier *et al.*, 1997; Mercier *et al.*, 1996). Increased mucus production has been previously noted in cnidarians exposed to heavy metals (Thompson *et al.*, 1980; Harland and Nganro, 1990) and it has been suggested that mucus plays a role in the regulation of metal concentrations of cnidarians by provision of binding sites (Harland and Nganro, 1990) as noted previously in discussions of responses to copper exposure. Increased mucus production will incur energetic costs to the host and consequently less energetic reserves will be available for fundamental biological processes such as reproduction or growth.

Morphological responses of *A. pallida* to arginine analogues

Contraction responses shown in *A. pallida* treated with arginine analogues are in line with the findings of previous studies that have used invertebrate tissue preparations (Elphick and Melarange, 1998). These authors attribute the observed contraction response to inhibition of the relaxing action of NO (Elphick and Melarange, 1998). Further work is needed to investigate the specificity of the contraction response observed in *A. pallida* exposed to arginine analogues. Pilot studies conducted here indicated that the contraction response was not abolished by co-injection of arginine analogues with an excess of arginine. These findings imply that the elicited effects are not exclusively the result of inhibition of NOS

activity. It is likely that the observed contraction of anemones may have been at least partly due to a toxicity response. Arginine analogues contain functional groups such as alcohols, amines and carboxylate groups that can interact with iron to form chelates of varying stability (Peterson *et al.*, 1992). Owing to the ubiquitous nature of iron in biological systems, compounds that interact with iron probably have several physiologic effects, e.g. L-arginine analogues have been reported to inhibit mitochondrial electron transport (Kiechle and Malinski, 1993). L-NNA generally elicited greater contractions in *A. pallida* than equivalent concentrations of L-NAME. This may be due either to a higher potency or toxicity of L-NNA, or differences in the uptake kinetics of the two analogues. The zooxanthellae observed in the experimental dishes containing *A. pallida* incubated for three hours in 5 mM L-NNA indicates disruption of the symbiotic association between host and zooxanthellae. Zooxanthellae loss is a well-documented response to adverse stimuli and is discussed further in chapter six.

In contrast to the observed contraction of *A. pallida* shown in response to injection with L-arginine alone (50 or 100 μ M), addition of exogenous L-arginine (0.1 mM and 1 mM) to cardiac stomach of the starfish was reported to caused relaxation (Elphick and Melarange, 1998) presumably due to enhanced NO production. The difference in the responses to treatment with exogenous arginine may be due to a range of factors including differences in biological preparation type (whole anemones versus dissected stomach preparations) and the mode of administration of chemical (direct injection into body cavity versus bath immersion).

Morphological responses to exposure to an exogenous NO donor

Results of the pilot study of the effects of exogenous NO on whole anemones showed a discrepancy in response to the two concentrations tested; *A. pallida* incubated in 20 μ M SNP contracted, while those incubated in 100 μ M expanded slightly more than LNSW

incubated controls. Both contraction and expansion have previously been reported in invertebrates (whole organisms or dissected organs) treated with NO donors; body contraction and tentacle shortening have been previously reported in hydra treated with 10 μ M SIN-1 (Colasanti *et al.*, 1997). In contrast, the relaxing effect of NO on smooth muscle has been observed in a variety of vertebrate preparations (Elphick and Melarange, 1998) and application of 10 μ M *S*-Nitrosoglutathione (SNOG) or *S*-Nitroso-*N*-acetyl-D, L-penicillamine (SNAP) (both NO donors) resulted in relaxing effects in starfish cardiac stomachs (Elphick and Melarange, 1998).

Overview of physiological responses

No link between NOS activity and morphological status is apparent in the results of the studies reported here. As such, no evidence was obtained that supports the hypothesis that NO is involved in regulating the morphological status of anemones.

A number of morphological changes were noted in *A. pallida* in response to the chemicals and contaminants tested including: contraction of tentacles and column, tentacle withdrawal, column base enlargement and detachment of pedal disc. Monitoring oral disc diameters was found to be an unsuitable monitoring tool as accurate measurement of this parameter was not possible when the anemones were contracted and/or the tentacles were withdrawn. It has been previously reported that anemone contraction can be provoked by almost any strong stimulus that might be considered unfavourable (Pearse, 1974) including application of predatory stimuli (Edmunds *et al.*, 1976) and an array of environmental factors (Bohn 1908, cited in Pearse, 1974). Past research has also reported that anemones continue to alter their expansion/contraction status in the apparent absence of any environmental stimuli (Batham and Pantin, 1950a; Needler and Ross, 1958); this may account for the changes in size shown by control anemones. The functional significance of alterations in the degree of expansion/contraction in *A. pallida* has not been

comprehensively documented but it has been suggested that periodic contractions of anemones may play a role in the circulation and exchange of coelenteric fluid (Needler and Ross, 1958). This may explain why morphological changes are observed in the apparent absence of adverse environmental stimuli (i.e. the size reductions observed by control anemones). The specific triggers for the acute contractions exhibited by *A. pallida* in response to the contaminants and chemicals tested in this research are not known but they may have been caused by specific biochemical interactions or the result of a general stress response. Specific information on the mechanisms by which morphological changes are brought about in the species *A. pallida* is lacking but the anemone *Metridium senile* has been well studied in this regard (Batham and Pantin 1950a, 1950c and 1953). It is likely that the mechanisms responsible for bringing about changes in the morphological status of anemones are similar in other members of class anthozoa. Changes in column shape of *Metridium senile* are brought about by reciprocal contractions of the circular muscle sheet, and of the longitudinal parietal musculature that runs at the junction of each mesentery with the body wall (Batham and Pantin, 1950a). The muscular systems of the mesenteries have been reported to be responsible for rapid responses to strong stimuli in *Metridium senile* (Batham and Pantin, 1950b). Tentacle contractions are presumably also brought about by muscular action. The content and distribution of hydraulic fluid, and internal hydrostatic pressure will be influenced by alterations in the degree of expansion or contraction. The column wall of *A. pallida* possesses small pores or cinclides (figure 1.4) through which water can be ejected from the central body cavity when the animal contracts (Edmunds *et al.*, 1976). Enlargement of the column base noted in anemones exposed to fluoranthene or the acetone carrier alone is likely to be a result of a change in the distribution of the body fluids brought about by contraction of the upper column and/or tentacles. Complete contraction of anemones requires expulsion of water from the pharynx; inversion of the oral disc, complete with tentacles into the pharynx may occur in extreme responses (Edmunds *et*

al., 1976). Rapid contraction of anemones when the mouth is open results in the expulsion of fluid from the gastrovascular cavity; responses such as these were elicited in anemones exposed to fluoranthene and TBTO at concentrations of 1000 or 100,00 µg/l. This form of response may represent a means by which anemones eliminate potentially harmful contaminants taken up from the environment into their gastrovascular cavity.

As outlined in the introduction to this chapter, alteration of the morphological status of anemones will affect a number of physiological parameters. Principally, changes in the degree of contraction and expansion of the column and oral disc, along with the extension and retraction of the tentacles can radically alter the area of gas exchange, the diffusion distance (Shick, 1991; Benazet-Tambutte and Allemand, 1997) and the surface area exposed to ambient conditions. In fully expanded anemones, the ratio of body surface area to body mass is maximal and the thickness of the body wall is minimal. Retraction of tentacles and column, and contraction of the oral disc have the opposite effect on these parameters. Contraction and expansion of anemones enforce great changes in thickness of the mesoglea (Batham and Pantin, 1950c). As sea anemones are soft-bodied organisms sustained by a hydrostatic skeleton, they are in direct contact with the surrounding seawater (Mercier *et al.*, 1998). As a result they are intimately exposed to, and hence vulnerable to contaminants present in their environment. The uptake of chemicals by cnidarians is likely to take place across the body surface as has been proposed for the uptake of metals (Harland and Nganro, 1990). Changes in the expansion status of sea anemones affects both the surface area and the thickness of the body wall, i.e. the available surface for the diffusion of molecules and the diffusion distance (Benazet-Tambutte and Allemand, 1997). By contracting, anemones simultaneously reduce their surface area and increase the diffusion distance. By this means it is possible that anemones reduce the uptake of chemicals from the environment; contraction responses such as those observed in anemones exposed to copper, fluoranthene or TBTO are therefore likely to represent a means by which anemones minimise exposure

to unfavourable conditions. It has been previously suggested that tight retraction of tentacles might restrict entry of metals (Harland and Nganro, 1990). Recent research has however reported that the permeability coefficients in contracted and relaxed tentacles of *Anemonia viridis* were similar (Benazet-Tambutte and Allemand, 1997). The authors interpreted these results as showing that the diffusional barrier is due to the water content of the mesoglea rather than the cellular junction. These authors also noted that the water permeability of the oral epithelial layers of *A. viridis* is relatively low. Further work is recommended to investigate the relationship between morphological status and contaminant uptake.

Anemones reduce the incidence of illumination by reducing their surface area by contracting; this is particularly significant in anemones that harbour photosynthetic symbionts. Expansion and contraction behaviour represents a means of regulating the amount of light reaching zooxanthellae (Pearse, 1974) and the host can regulate the degree of photosynthetic activity by tentacle contraction (Glider *et al.*, 1980). Photosynthetic rates are likely to alter as a result of changes in the degree of expansion/contraction, with reduced rates expected in contracted anemones. As the cnidarian symbiotic hosts are partially nutritionally dependent on translocated photosynthate, alterations in rates of photosynthesis may have implications for the nutritional status of the host. In addition to these possible nutritional implications, zooxanthellae have been shown to be important in the uptake of a variety of chemicals (Pearse, 1974; Mercier *et al.*, 1996; Denton and Burdon-Jones, 1986; Harland and Nganro, 1990; Harland *et al.*, 1990) and as previously noted, levels of illumination and rates of photosynthesis have been shown to influence the uptake of butyltin in *A. pallida* (Mercier *et al.*, 1996). Thus contraction responses exhibited by anemones exposed to copper, fluoranthene or TBTO may have been elicited in an attempt to minimise contaminant uptake by both reducing the exposed surface area and by reducing uptake by the zooxanthellae.

Contracted anemones reduce or cease to exhibit feeding responses (personal observation). Despite contributions made by zooxanthellae to the nutrition of the host, periodic meals are necessary to maintain nutritional sufficiency (Mercier *et al.*, 1996). Additionally, nutrient deficiencies have been noted in zooxanthellae contained in anemones that have ceased to feed (Cook *et al.*, 1988; Cook *et al.*, 1992). Part of the nutritional requirement of cnidarians is provided by the diffusion of molecules from the environment. Owing to the reduced surface area and increased diffusion distance of contracted anemones, it is likely that this source of nutritional supply is reduced or absent in contracted anemones. Contraction behaviour is therefore likely to have detrimental implications for both the nutritional status and energetic reserves of the symbiosis; this may ultimately result in adverse impacts on organismal fitness parameters such as growth and reproductive output.

Detachment of anemones was noted in anemones exposed to copper or fluoranthene and in the control anemones used in these experiments. As mentioned previously, detachment may have been caused by anemones responding to the gentle agitation applied to the experimental dishes during these experiments. This theory is supported by the observation that no detachment was observed in anemones exposed to TBTO and the respective controls that were not agitated during exposures. Detachment of anemones was however noted in the absence of agitation in anemones exposed to 5 mM L-NNA. In this instance it is likely that detachment may be explained by the acute contractions exhibited by these individuals. The natural habitat of *A. pallida* is in sheltered bays and lagoons as outlined in chapter one. Water and substrate (such as mangrove roots and sponges) movement in habitats such as these are likely to be minimal. For this reason agitation of anemones applied during experimentation may have been deemed to be unfavourable. As such, the anemones may have detached in an attempt to relocate to more favourable conditions.

NO and morphological status

Although no clear relationship was apparent between NOS activity and morphological status in *A. pallida*, the involvement of NO in physiological responses of anemones cannot be ruled out on the basis of these preliminary findings. NO is a messenger molecule that rapidly reaches and acts on neighbouring target cells (Johansson and Carlberg, 1995), as such it is a possible elicitor of the fast contractile response of anemones to a range of stimuli. It is possible that the experimental approach used here, specifically the use of whole organism preparations, was not sensitive enough to detect alterations in NOS activity relating to a signalling role.

Further investigations of the morphological changes resulting from contaminant exposure are recommended. Recognition and definition of sublethal and lethal endpoints of toxicity induced morphological changes in *A. pallida* would enable physiological monitoring to be used in toxicity assessment. To assist this, an improved monitoring approach to contaminant induced morphological changes is recommended using time-lapse photography (as pioneered by Batham and Pantin i.e. 1950c) or by continuous video filming. In parallel with studies of this kind, the implications of contractions for the uptake, accumulation and elimination of contaminants should be quantified and the impact of morphological changes on a range of measures of organismal fitness should be assessed.

Limitations of results and suggestions for future work

The results presented here are from preliminary studies and as a consequence a number of limitations apply to the findings: Perhaps of greatest importance was the failure to quantify actual exposure concentrations of contaminants used in each experiment. It is appreciated now that failure to this was a serious oversight of experimental design, with possible profound implications for all the results obtained. Knowledge of the actual concentration of contaminants or chemicals the anemones were exposed to, combined with knowledge of the

bioavailability, uptake, accumulation and elimination of contaminants, is essential to facilitate a full understanding of the observed biological responses. Each of the studies exposed anemones to single contaminants for short periods (maximum 72 hours). While studies of this kind are convenient for pilot studies, they are generally unrepresentative of actual environmental exposures. This limitation could be addressed by monitoring a range of appropriate parameters during longer-term exposures to environmentally realistic, sublethal concentrations of contaminants. Morphological alterations of anemones, along with mucus production and changes in zooxanthellae densities are likely to have influenced contaminant uptake. Measurement of body burdens of contaminants is recommended for future studies; this would enable the relationship between contaminant exposure and uptake, and the elicited effect to be better interpreted.

Despite noted limitations of these studies, the results contribute to knowledge of cnidarian contaminant responses and provide information that can be used cautiously as the basis of further studies. Although no evidence was provided to support the hypothesis that NOS is a suitable biomarker of exposure of *A. pallida* to copper, fluoranthene or TBTO, the role of NO in contaminant responses merits further study. Recent research has identified NO to be among a number of intracellular redox signals that are capable of triggering a series of gene events that ultimately result in the activation of a gene that confers resistance to oxidants, antibiotics, solvents and some metals, (Hidalgo *et al.*, 1997). These findings link cellular oxidative stress, and in particular NO to the expression of defence genes (Hidalgo *et al.*, 1997). As oxidative stress is a well-recognised consequence of xenobiotic exposure (Winston *et al.*, 1991), involvement of NO in the triggering of protective cellular effects warrant further investigation. The future for cnidarian biomarker research may be in differential display polymerase chain reaction (PCR) used to identify toxicant-induced genes that can serve as molecular biomarkers for rapid toxicity assessment (Morgan and Snell, 2000, Morgan *et al.*, 2001).

Further studies using arginine analogues and NO donors with appropriate controls are needed to elucidate the role of NO in *A. pallida*. The specificity of the contraction response could be investigated by use of D-enantiomers of NAME and NNA. In these studies some effects due to the action of an isomerase that converts D-arginine to L-arginine (Elphick and Melarange, 1998) can be expected but it may be possible to determine the degree of specificity of the elicited response. As rates of citrulline formation were inhibited in a dose dependent manner by L-NMA (chapter 3) it would be of interest to investigate the morphological response of whole anemones to a range of concentrations of this analogue. Further investigations of the response of anemones to NO donors are suggested. As reduction of SNP and the subsequent release of the nitrosyl complex are accompanied by cyanide release (Feelisch and Stamler, 1997a), use of controls treated with the cyanide anion are recommended for future studies. The results provided by this study can be used as a foundation for future studies using a wider selection of compounds, concentration ranges and incubation periods.

CHAPTER SIX
CNIDARIAN –
SYMBIODINIUM
SYMBIOSES,
BLEACHING AND
OXIDATIVE STRESS

Chapter Six

Cnidarian-Symbiodinium Symbioses, Bleaching **and Oxidative Stress**

The current paradox in coral reef research is that the organisms and ecosystems involved seem to be vulnerable to environmental change in the short term, yet they have persisted over hundreds of millions of years, through rapid environmental change (Buddemeier, 1997).

Cnidarian bleaching

The term 'bleaching' was first used by Glynn in 1983 to describe the phenomenon in which symbiotic scleractinian corals lose coloration due to loss of symbiotic zooxanthellae or zooxanthellae pigments. Bleaching has been observed in a variety of cnidarians, including anemones and zoanthids, as well as in molluscs, sponges, ascidians and large foraminifers in symbiotic association with diatoms, dinoflagellates or cyanobacteria (Tsimilli-Michael *et al.*, 1999). Cnidarian bleaching represents a breakdown of the intimate and highly regulated association between host (cnidarian) and symbiont zooxanthellae by, an as yet uncharacterised mechanism. Bleaching events are dramatic phenomenon involving loss of large amounts of zooxanthellae and/or chlorophyll. These events are characterised by partial or total loss of coloration in the host cnidarian. It has been documented that corals containing reduced zooxanthellal densities grow slowly if at all, are more susceptible to disease and have a reduced ability to remove sediment and regenerate injured tissue (Mascarelli and Bunkley-Williams, 1999). Recovery can occur after a bleaching incident either by reacquisition of a stock of zooxanthellae (either from external sources or from replication of the residual stock of zooxanthellae) or by increased pigment levels in retained zooxanthellae. Prolonged bleaching commonly results in coral mortality. A wide range of biological phenomena have been identified in both host and symbiont prior to, during and following natural and artificially induced bleaching events (detailed in section 1.7). To date,

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only a few experimental studies of the cellular or molecular mechanism of cnidarian bleaching (Nii and Muscatine, 1997) have been conducted. Recent work has implicated the following in bleaching events: photosynthetic disruption, (damage to, or declines in the efficiency of photosystem II) (Warner *et al.*, 1999; Jones and Hoegh-Guldberg, 1999), production of active forms of oxygen (Lesser, 1997; Lesser, 1998; Lesser, 1990; Lesser, 1996), and elevations of intercellular calcium concentrations (Huang *et al.*, 1998).

Mechanisms of zooxanthellae density reduction

A range of mechanisms that result in zooxanthellae density reductions have been reported including: exocytosis of zooxanthellae from the host cell which results in the release of isolated algae (Steen and Muscatine, 1987), cell death due to both apoptosis and necrosis (Searle *et al.*, 1982), pinching off of the distal portion of the host cell (Glider, 1980) and detachment of host cells (Gates *et al.*, 1992). It is thought likely that no single mechanism of release operates exclusively (Brown *et al.*, 1995).

Bleaching triggers

In general, the disassociation of algal-invertebrate symbioses appears to be in response to extremes in environmental conditions (Iglesias-Prieto *et al.*, 1992). Numerous triggers of bleaching events have been identified including: temperatures outside the normally encountered range, high doses of UV, bacterial infection, changes in salinity, aerial exposure, and exposure to a wide range of 'anthropogenic stresses'. Ascribing specific causes to bleaching events has proved to be largely elusive as a result of the extreme complexity and heterogeneity of the shallow reef community environment, the technical limitations of continuous environmental monitoring, and the failure of laboratory studies to adequately imitate field conditions (Brown, 1997). Bleaching appears to be a generalised

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response caused by many different stressors (Bastidas *et al.*, 1992), which singly, or in combination disrupt the symbiotic equilibrium sufficiently for it to breakdown. For an extensive review of the physical and biological factors involved in bleaching, the mechanisms of zooxanthellae release, and the ecological consequences for coral communities the reader is directed to Brown (1997).

Elevated water temperature is the single most documented environmental parameter associated with coral bleaching. Abnormalities of as little as 1-2°C have been documented in bleaching episodes (Fitt and Warner, 1995). Bleaching events are characterised by locally defined upper thermal limits; corals in habitats that are more variable or more prone to stress tend to bleach less than those in more equable environments (Buddemeier and Fautin, 1993). In addition, there are consistent taxonomic differences in vulnerabilities to stress-related bleaching and associated mortality at a particular site (Cook *et al.*, 1990). Bleaching has been hypothesised to be an adaptive mechanism that ultimately allows zooxanthellae and corals to exchange symbiotic partners and thereby compensate for environmental stresses (Buddemeier and Fautin 1993), but little evidence has thus far been provided to support this. Sea temperatures in many tropical regions have increased by almost 1°C over the past 100 years, and are currently increasing at ~1-2°C per century (Hoegh-Guldberg, 1999). As a result of the link between elevated sea temperature and coral bleaching events, predictions of further rises are cause for serious concern for the future health and survival of the world's coral reef communities.

Characteristics of recent bleaching events

Evidence exists that coral bleaching events have increased dramatically since the early 1980's (Glynn, 1993). In the period 1876-1979, 3 major bleaching events were reported compared to 60 in the period 1979-1990 (Glynn, 1993). The high frequency and intensity of

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recent bleaching events has resulted in extensive degradation of coral reefs throughout the world (Meehan and Ostrander, 1997). It has been suggested that human induced alterations to the natural environment may be accelerating 'normal' bleaching events (Meehan and Ostrander, 1997).

Oxidative stress

A consequence of all aerobic life is the production of potentially harmful, partially reduced species of molecular oxygen (oxyradicals) that occur as a result of normal oxygen metabolism (Winston and Di Giulio, 1991). Cellular respiration involves the reduction of molecular oxygen to water in the electron transport chain (Kelly *et al.*, 1998). Several additional oxidising enzymes produce reactive oxygen species (ROS), comprising a second source of ROS within cells (Kelly *et al.*, 1998). The term ROS is used in preference to oxygen radicals since H_2O_2 and singlet O_2 and $HOCl$ are non-radicals (Halliwell, 1994b). In addition to endogenously generated oxidants, organisms are exposed to prooxidants arising from natural products in dietary sources and from environmental pollutants including trace metals and organic compounds that are known to enhance the formation of ROS (Regoli and Winston, 1998). Cellular damage caused by ROS is commonly referred to as oxidative stress (Gamalay and Klyubin, 1999).

In view of the potential for harm, aerobic organisms have evolved defences to protect themselves against the possible effects of ROS production (Winston and Di Giulio, 1991). These defences are collectively referred to as antioxidants and are defined as; substances that, when present at low concentrations compared with those of an oxidisable substrate, significantly delay, reduce or prevent oxidation of that substrate (Halliwell, 1994b). The antioxidant defence mechanism is comprised of primary antioxidants (vitamins, micronutrients and enzymes such as superoxide dismutase (SOD) and catalase), secondary

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antioxidant defences (e.g. repair enzymes) and, thirdly, oxidants (e.g. hydrogen peroxide) or processes leading to their formation such as redox cycling (Stegeman *et al.*, 1992).

Since oxidative stress levels may vary over time, organisms are able to adapt to these fluctuating stresses by inducing the synthesis of antioxidant enzymes and damage removal/repair enzymes (Rice-Evans and Halliwell, 1994). As a result of this, variations in endogenous levels of antioxidants in marine organisms have been proposed as useful biomarkers in monitoring studies (Winston and Di Giulio, 1991). Oxidative damage or oxidative stress occurs when there is an imbalance in the generation and removal of radical species within an organism (Kelly *et al.*, 1998). Oxidative stress appears to be the basis for a wide array of physiological aberrations (Winston and Di Giulio, 1991); the toxic consequences of oxidative stress at the subcellular level include lipid peroxidation and oxidative damage to DNA and proteins (Kelly *et al.*, 1998).

Chemistry of oxidative stress and free radicals

The paradox of aerobic life, or the 'oxygen paradox' is that higher eukaryotic aerobic organisms cannot exist without oxygen, yet oxygen is inherently dangerous to their existence (Davies, 1994). The basis of this paradox is that one-electron reduction of O₂ generates the superoxide anion free radical O₂^{-•}, from numerous biological sources (Ahmad, 1997). In all biological systems O₂^{-•} undergoes further reduction to H₂O₂ via the Fenton reaction to the hydroxyl radical, •OH (equation 6.1).



The term ROS is used to indicate products generated as intermediates in the redox (oxidation-reduction) processes leading from molecular oxygen to water; any species that is

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more reactive than the ground state oxygen molecule but does not react directly (by a non-catalytic mode) with most organic substances is an ROS (Gamalay and Klyubin, 1999). The most reactive forms of ROS known are the hydroxyl radical's $\cdot\text{OH}$ and $^1\text{O}_2$ (Ahmad, 1997). The relative importance of antioxidants as protective agents depends upon which ROS is generated, how it is generated, where it is generated, and what target of damage is measured (Halliwell, 1994b). Among the deleterious reactions of ROS are: oxidation of proteins, DNA and steroidal compounds, and peroxidation of the cell membrane's unsaturated lipids leading to lipid peroxidation (Ahmad, 1997).

Bleaching, oxidative stress and nitric oxide

One hypothesised mechanism of bleaching involves an increase in the production of reactive oxygen species (ROS) in the dinoflagellate symbionts and host tissues sufficient to overcome the endogenous antioxidant defence mechanisms of the tissues, resulting in cellular damage and expulsion of symbionts (Lesser, 1997). The continuous flux of photosynthetically produced hyperbaric oxygen in the tissues of *A. pallida* is countered by a high antioxidant capacity and it is known that host superoxide dismutase activity is altered in response to the amount of oxygen generated by its intracellular algal symbionts (Dyken and Shick, 1982). Antioxidant defences of both host and zooxanthellae are likely to be involved in protecting against ROS induced damage.

NO has been shown to participate in numerous physiological processes (Snyder and Bredt, 1992). The formation of NO can lead to the production of reactive nitrogen species that can result in damage to specific cellular targets. Paradoxically, NO has been shown to protect against oxidative stress mediated by ROS by stimulating expression of protective proteins or by affecting other cellular functions which may attenuate oxidative stress (Wink *et al.*, 1999). Thus endogenous NO may generate either prooxidant or antioxidant conditions

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depending on the availability and concentration of potential reaction partners such as superoxide and hydrogen peroxide or other reactive oxygen species (ROS) (Beck *et al*, 1999).

As elevated temperature is the major factor attributed to the induction of bleaching events, preliminary laboratory studies were designed to investigate the response of NOS activity in whole anemone or cytosolic preparations of *A. pallida* exposed to short-term heat shock. Following this work, further experiments were designed and conducted to investigate the relationship between NOS activity and anti-oxidant status during a thermally induced bleaching event. Bleaching was assessed by monitoring zooxanthellae densities and chlorophyll a and c₂ concentrations.

6.2 Materials and methods

Experiments were designed to investigate the physiological response of whole anemones and anemone cytosol to short term heat shock, and whole anemones to longer-term exposure to increased ambient temperatures. Anemones used in experimentation were maintained in standard incubation conditions prior to use (section 2.1). A temperature regime was designed to be environmentally relevant, sublethal and to evoke bleaching over time. Some of the temperatures tested were outside the normally encountered temperature range of the collection site (as shown in table 2.1), but were representative of either the thermal extremes experienced by Bermuda's near shore coral reefs or had been documented in past cnidarian research (i.e. Black *et al.*, (1995) heat stressed *A. pallida* at temperatures up to 35°C).

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Short-term exposure to elevated temperature

Anemone cytosol from a single preparation was incubated at either 25°C or 33°C for two hours. In other experiments, whole anemones (six-nine individuals per treatment) were exposed to 33°C for two or four hours prior to determination of rates of citrulline formation in host cytosolic aliquots. Control anemones were maintained at 25°C for the experimental duration. Anemones were set-up in glass culture dishes with 70 ml LNSW. Anemone tissue from each treatment was collectively homogenised and prepared for determination of host NOS activity.

Longer term exposure to elevated temperatures

Background levels of citrulline generation (section 2.2) antioxidant activity (via the ferric reducing/ antioxidant power (FRAP) assay (section 2.3)), chlorophyll a and c₂ content (section 2.8), zooxanthellae densities (section 2.7) and protein content (section 2.5) were determined in 10 individual anemones prepared separately. Chlorophyll a is the dominant photosynthetic pigment in zooxanthellae and it is commonly quantified in symbiotic cnidarian studies (Cook *et al.*, 1988; Dykens and Shick, 1982; Muller-Parker, 1987; Lesser and Shick, 1989a; Jones *et al.*, 1998; Dykens *et al.*, 1992; Lesser, 1989; Kizner *et al.*, 1995). Anemones used for background determinations were collected from Walsingham Pond nine days prior to assay and were maintained in standard culture conditions until the start of the experiment (section 2.1). Another set of anemones, collected on this same occasion, was exposed to gradual increases in ambient temperature. A total of nine groups, each of six individuals were placed in 120 ml glass culture dishes with 70 ml LNSW. Culture dishes containing anemones were set up in a thermally controlled water bath set at 25°C. Culture dishes were uncovered to allow visual monitoring with minimum disturbance. Experimental dishes were cleaned and 70 ml fresh LNSW of an appropriate temperature added at 24-hour

intervals. Temperatures of the water bath and semi-immersed dishes were checked on a regular basis. The water bath was found to reflect the setting $\pm 0.5^{\circ}\text{C}$. The temperature of the LNSW within the culture dishes surrounding the anemones reflected those of the surrounding water bath with a lag time of approximately 2 hours. Water levels in culture dishes were checked frequently and maintained by addition of fresh LNSW of an appropriate temperature. The water bath liquid was monitored and maintained at a level just higher than the water level in the culture dishes. An initial period of 48 hours was allowed for the anemones to settle into the glass dishes. Temperature increases of 2°C were made each 24-hour period until 31°C was reached. After a total of five days (120 hours) at 31°C , temperatures were again raised by 2°C each 24-hour period until a maximum of 35°C was reached. The temperature regime is illustrated in figure 6.1.

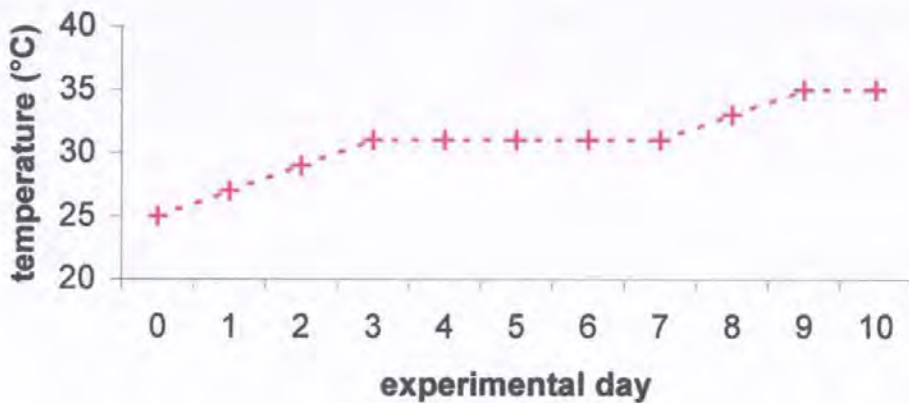


Figure 6.1. Thermal regime applied to whole anemones. Six individual anemones were sacrificed on days, 0, 1, 2, 3, 4, 5, 8, 9 and 10 and rates of citrulline formation, FRAP activity, chlorophyll a and c_2 concentrations, zooxanthellae densities, and the protein content of individual anemones was determined.

Prior to the application of temperatures above 25°C (experimental day 0) and on a further 8 time points over the course of the experimental period (days 1, 2, 3, 4, 5, 8, 9 and 10), six anemones from a single dish were sacrificed. Groups of anemones were subject to all preceding temperature conditions. The following parameters were determined in each: NOS

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activity (as measured by citrulline formation), antioxidant capacity (measured by the FRAP assay), zooxanthellae density, chlorophyll a and c₂ and protein content. Three groups (six individuals each) were maintained at 25°C for the duration of the experiment as physiological monitoring control animals. Total width and oral disc diameter (method as described in chapter five) of control and experimental anemones were measured each 24 hours. Experimental anemones were measured immediately before the water bath temperature was raised.

Individual anemones were homogenised as detailed in section 2.1. Aliquots of 200 µl homogenate were frozen (-20°C) for zooxanthellae density determination and chlorophyll extraction. The remaining homogenate was centrifuged at 16000 RCF for 10 minutes (at 4°C) and aliquots of supernatant (cytosol) removed for the FRAP and Citrulline assays (detailed in sections 2.3 and 2.2 respectively). Reproducibility of the FRAP and citrulline assays was established with multiple identical aliquots of anemone cytosol.

6.3 Results

Short-term exposure to elevated temperature

Rates of citrulline formation were increased in both cytosolic aliquots and whole anemones (six or nine individuals per treatment) exposed to temperatures of 33°C for two or four hours compared to controls maintained at 25°C. Rates of citrulline formation in heat exposed anemones or preparations were statistically different from those of controls maintained at 25°C ($p < 0.05$) (figures 6.2, 6.3 and 6.4). The increased rates of citrulline formation in heat shocked samples relative to controls varied between experiments and ranged from 23.0 to 77.4%; rates of citrulline formation in control anemones also varied between experiments.

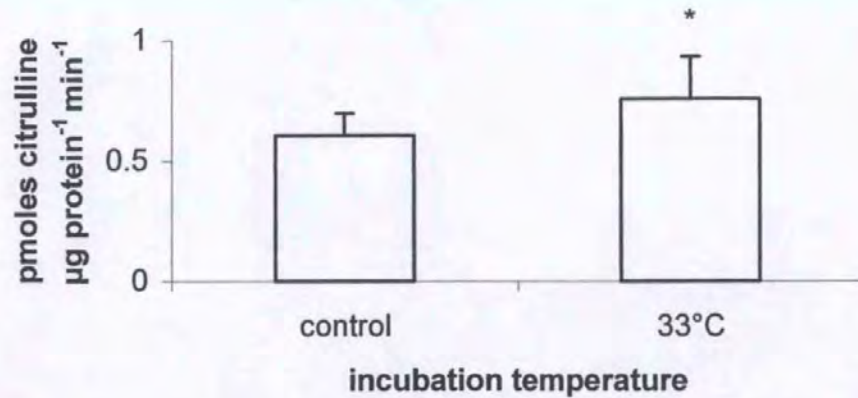


Figure 6.2. Rates of citrulline formation in cytosolic preparations of whole *A. pallida* maintained at 25°C (control) or incubated at 33°C for 2 hours. Nine anemones were exposed to each treatment. Values shown are means of triplicate determinations. Error bars show standard deviations; the asterisk indicates a value statistically significant different ($p < 0.05$) from control values.

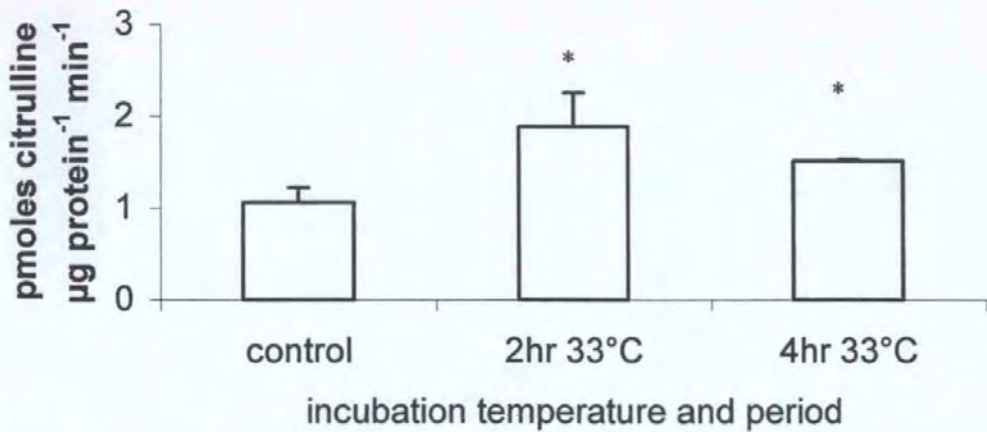


Figure 6.3. Rates of citrulline formation in cytosolic preparations from whole *A. pallida* maintained at 25°C (control), or incubated for two or four hours at 33°C. Six anemones were exposed to each treatment. Values shown are means of triplicate determinations. Error bars show standard deviations, asterisks indicate values statistically significantly different ($p < 0.05$) from controls.

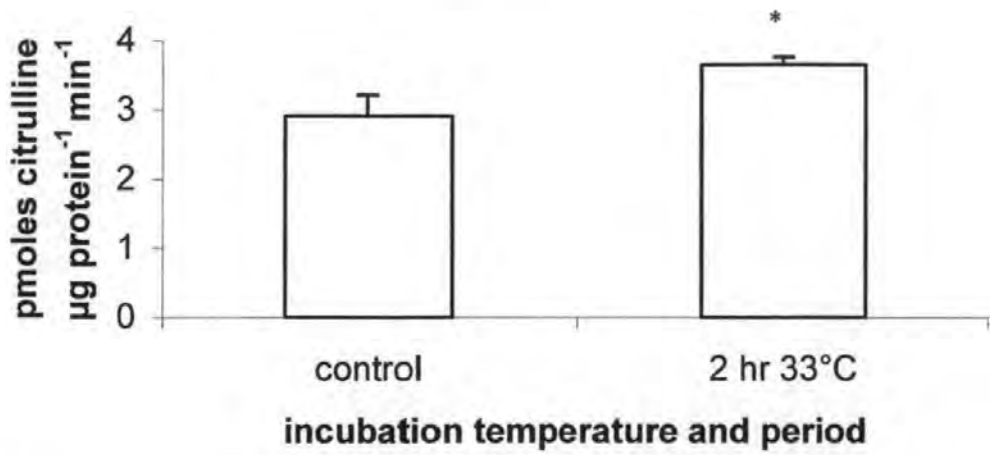


Figure 6.4. Rates of citrulline formation in *A. pallida* cytosol (aliquots of a single cytosolic preparation) pre-incubated for two hours at 25°C (control), or at 33°C for two hours. Values shown are means of triplicate determinations. Error bars show standard deviations, asterisks indicate values statistically significantly different ($p < 0.05$) from controls.

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FRAP intra assay variance

Acceptable reproducibility of the FRAP assay was demonstrated with 10 identical aliquots of anemone cytosol. An overall mean of 225 ± 13.8 nmoles/minute FRAP units was determined representing a % C.V. of 6.1. Note: regretfully the protein content of the cytosol used in these FRAP intra assay variance determinations was not quantified. As a result, the FRAP values are expressed as nmoles/minute.

Background levels of assay parameters

Background levels of citrulline generation; FRAP values, chlorophyll a and c₂ concentrations, zooxanthellae densities, and protein contents are presented in tables 6.1-6.5. Values shown for each parameter are the minimum and maximum determined from the 10 individuals assayed and, overall means and standard deviation calculated from the entire experimental group.

Background levels of citrulline formation ranged from 4.05-9.96 pmoles citrulline μg protein⁻¹ min⁻¹ (table 6.1). On a per anemone basis, the highest activity detected was over 8 times that of the lowest value.

	Minimum	Maximum	Mean	S.D.
Citrulline (pmoles μg protein ⁻¹ min ⁻¹)	4.05	9.96	6.71	1.84
Citrulline (pmoles anemone ⁻¹ min ⁻¹)	1.08×10^3	8.82×10^3	5.59×10^3	2.18×10^3

Table 6.1. Rates of citrulline formation in cytosol from 10 individual anemones each assayed in triplicate. Citrulline per anemone data was extrapolated from the combined citrulline assay results and calculations of the protein contents of whole anemones (derived from protein assay of cytosol and estimates (based on total cytosolic volumes) of the protein constant of individual anemones).

FRAP values detected in anemone cytosol ranged from 0.11-0.36 $\mu\text{M}/\text{mg}$ protein (table 6.2). Considerable variance in FRAP values calculated in terms of mirco-moles both in terms of protein content and per anemone was shown (table 6.2).

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	Minimum	Maximum	Mean	S.D.
FRAP value ($\mu\text{M}/\text{mg}$ protein)	0.11	0.36	0.27	0.08
FRAP value ($\mu\text{M}/\text{anemone}$)	0.04	0.51	0.25	0.15

Table 6.2. FRAP values of cytosol from 10 individual anemones. FRAP values were calculated using combined data from all FeSO_4 standard concentrations. FRAP per anemone values were extrapolated from the FRAP assay results and calculations of the protein contents of whole anemones (derived from protein assay of cytosol and estimates (based on total cytosolic volumes) of the protein content of individual anemones).

Despite selection of anemones of apparently similar sizes (based on observation of oral disc diameter and tentacle length), large variations in the protein contents of anemones assayed were found (table 6.3). The maximum protein biomass determined for an individual anemone was nearly six times that of the smallest biomass detected.

	Minimum	Maximum	Mean	S.D.
Protein ($\text{mg}/\text{anemone}$)	0.27	1.56	0.88	0.40

Table 6.3. Protein contents of anemone cytosolic preparations. Values shown are combined data for 10 individual anemones. Protein contents of whole anemones were derived from the results of protein assays of cytosol, combined with total cytosolic volumes of individual anemones.

The total number of zooxanthellae cells per anemone and per unit host protein ranged widely as shown in table 6.4. The highest zooxanthellae density per anemone determined (4.58×10^6) was close to 6 times higher than the lowest density (8.20×10^5). Zooxanthellae densities per unit protein ranged from 0.57-8.68 cells per mg host protein.

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	Minimum	Maximum	Mean	S.D.
Zooxanthellae Density (cells/ anemone)	8.20×10^5	4.58×10^6	2.19×10^6	1.12×10^6
Zooxanthellae cells/ mg host protein	0.57	8.68	3.07	2.27

Table 6.4. Zooxanthellae density values. Zooxanthellae densities were counted in small aliquots of homogenate from 10 individual anemones; estimates of number of zooxanthellae per anemone, or per unit protein were calculated by combining these results with total per-anemone homogenate volumes, or total anemone protein values respectively.

Chlorophyll a contents ranged between 5.15 and 72.6 pg/ zooxanthellae cell and between 8.81 and 71.46 μg chlorophyll/ μg host protein (table 6.5).

Tables 6.1-6.5 show that there was considerable inter-anemone variance in each of the assessed parameters.

	Minimum	Maximum	Mean	S.D.
Chlorophyll a (pg/ zooxanthellae)	5.15	72.6	19.6	19.0
Chlorophyll c_2 (pg/ zooxanthellae)	1.46	17.6	5.05	4.51
μg chlorophyll a/ μg host protein	0.01	0.06	0.04	0.01
μg chlorophyll c_2 / μg host protein	0.003	0.016	0.01	0.003
Total μg Chlorophyll a/ anemone	8.81	71.46	35.22	19.74
Total μg Chlorophyll c_2 / anemone	2.09	19.30	9.42	5.29
Ratio a: c_2 (μg / anemone)	3.05	4.22	3.78	0.35

Table 6.5. Chlorophyll data from analysis of 10 individual anemones. Chlorophyll data (both a and c_2) is expressed as: μg per zooxanthellae cell, per μg host protein, total μg per anemone and as a ratio of total μg of each a and c_2 .

Responses to long term exposure to elevated temperatures

Rates of citrulline formation

Rates of citrulline formation in cytosol from anemones exposed to temperatures between 25-31°C ranged between 3.6-5.4 pmoles μg protein⁻¹ min⁻¹ (n=36) (figure 6.5). An overall mean of 5.0 ± 1.6 pmoles μg protein⁻¹ min⁻¹ was calculated from combined data from this

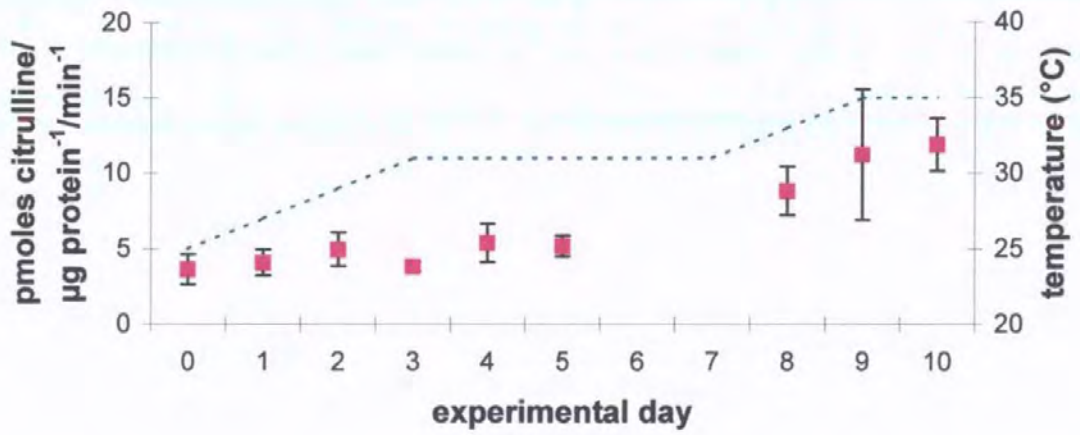


Figure 6.5. Rates of citrulline formation in cytosolic preparations of whole *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day, each run in triplicate; error bars show standard deviations.

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period; this mean was used as a baseline for comparison with other experimental values. Protein normalised rates of citrulline formation ($\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$) increased in response to temperatures above 31°C (figure 6.6). Anemones exposed to 33°C for 24 hours had rates of citrulline formation equal to $8.8 \pm 1.6 \text{ pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$, indicating a rate increase of 95.6% from baseline levels. Rates of citrulline formation in anemones exposed to 35°C were further increased. Rates of $11.2 \pm 4.3 \text{ pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$ and $11.9 \pm 1.8 \text{ pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$ after 24 and 48 hours respectively were recorded. Citrulline formation values calculated on a per animal basis ranged from $4.61\text{--}9.88 \times 10^3 \text{ pmoles anemone}^{-1} \text{ min}^{-1}$ (mean $7.12 \times 10^3 \pm 1923$) ($n=54$) over the entire course of the experiment (figure 6.7). No clear pattern of change in rates of formation per anemone was evident.

Total antioxidant capacity

ANOVA analysis revealed statistically significant differences ($p < 0.00005$; d.f. = 8) between the mean FRAP values of 17 pairs of anemone groups, from nine thermal treatments. The greatest estimated difference was found between groups of anemones exposed to 24 hours at 29°C and those exposed to 35°C for 48 hours (table 6.6).

Anemones maintained at between 25°C and 31°C had FRAP values ranging from 0.17 to $0.28 \text{ } \mu\text{M/mg protein}$ (mean 0.30 ± 0.16 ($n=36$)) (figure 6.8). Incubation of anemones at temperatures above 33°C resulted in increased protein normalised cytosolic FRAP values compared to anemones maintained at temperatures $\leq 31^{\circ}\text{C}$ (figure 6.9). FRAP values of anemones exposed to 33°C for 24 hours, or 35°C for 48 hours were different statistically ($p < 0.05$) from those detected in anemones exposed to between 25 and 31°C . After 24 hours exposure to 33 or 35°C , FRAP levels were $0.35 \pm 0.08 \text{ } \mu\text{M/mg protein}$ and $0.38 \pm$

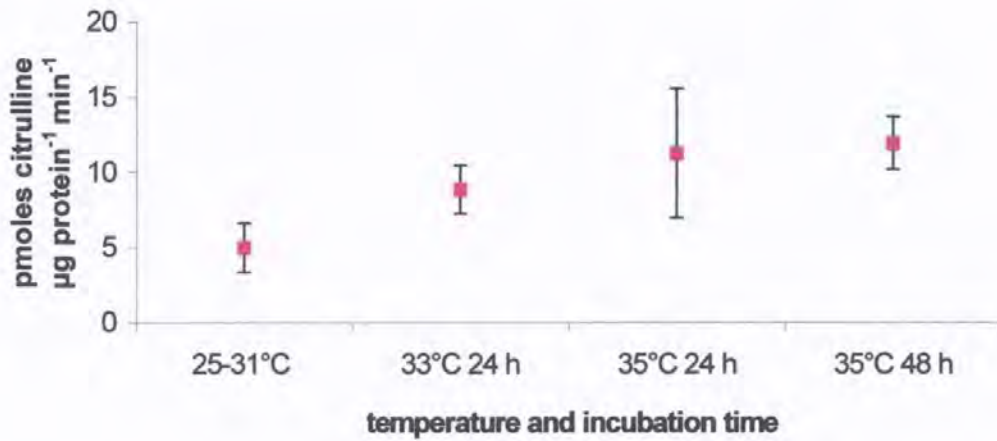


Figure 6.6. Rates of citrulline formation in cytosolic preparations of whole *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours. Values shown are means of six anemones per treatment (apart from the 25-31°C value which is a mean of 36 anemones exposed to temperatures in this range for between 24 and 72 hours) each run in triplicate; error bars show standard deviations.

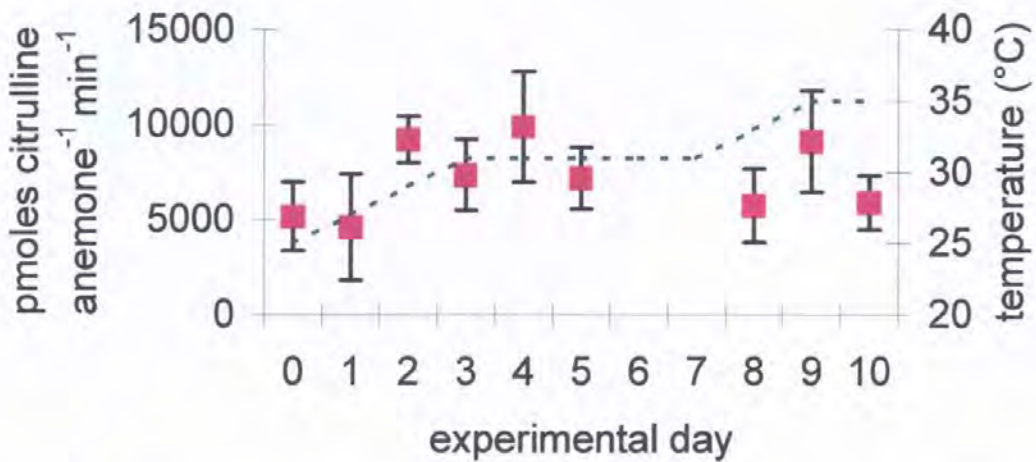


Figure 6.7. Rates of citrulline formation per anemone determined from cytosolic preparations of whole *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

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Treatments compared		Difference between treatments
48 hours, 25°C (0)	24 hours, 35°C (9)	1.25×10^{-4}
48 hours, 25°C (0)	48 hours, 35°C (10)	3.72×10^{-4}
24 hours, 27°C (1)	24 hours, 29°C (2)	1.17×10^{-4}
24 hours, 27°C (1)	48 hours, 35°C (10)	3.44×10^{-4}
24 hours, 29°C (2)	24 hours, 33°C (8)	1.80×10^{-4}
24 hours, 29°C (2)	24 hours, 35°C (9)	2.13×10^{-4}
24 hours, 29°C (2)	48 hours, 35°C (10)	4.60×10^{-4}
24 hours, 31°C (3)	24 hours, 35°C (9)	1.40×10^{-4}
24 hours, 31°C (3)	48 hours, 35°C (10)	3.87×10^{-4}
48 hours, 31°C (4)	24 hours, 33°C (8)	1.14×10^{-4}
48 hours, 31°C (4)	24 hours, 35°C (9)	1.47×10^{-4}
48 hours, 31°C (4)	48 hours, 35°C (10)	4.00×10^{-4}
72 hours, 31°C (5)	24 hours, 33°C (8)	1.60×10^{-4}
72 hours, 31°C (5)	24 hours, 35°C (9)	1.90×10^{-4}
72 hours, 31°C (5)	48 hours, 35°C (10)	4.40×10^{-4}
24 hours, 33°C (8)	48 hours, 35°C (10)	2.80×10^{-4}
24 hours, 35°C (9)	48 hours, 35°C (10)	2.48×10^{-4}

Table 6.6. Results of comparison of mean FRAP values in pairs of experimental groups of anemones ($n=6$, \pm limits 1.09×10^{-4}). Differences are shown for group pairs (displayed in rows) with means determined to be statistically different from one another at the 95% confidence interval. Experimental days corresponding to each treatment are shown in parentheses. Treatments were accumulative (i.e. anemones analysed after exposure to 24 hours at 27°C had been exposed to the preceding thermal regime (detailed in figure 6.1)).

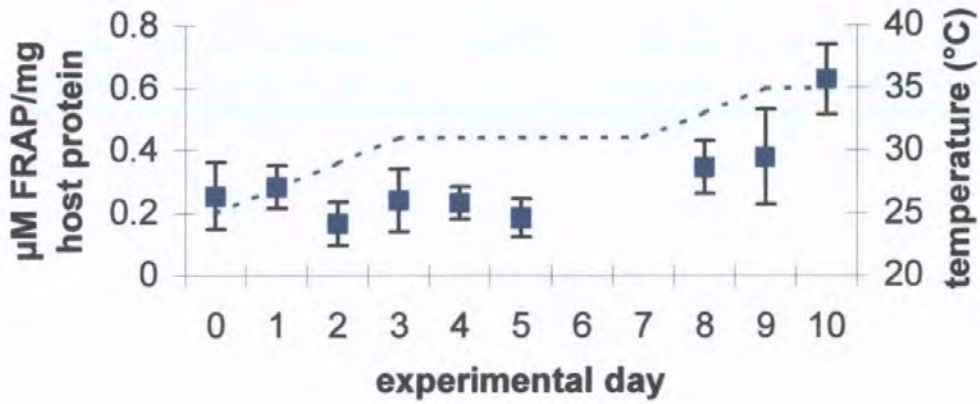


Figure 6.8. FRAP activity in host cytosol of *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

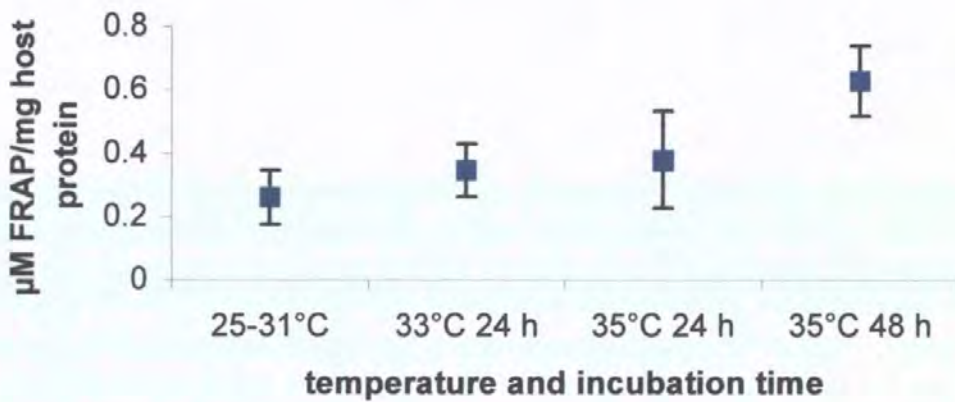


Figure 6.9. FRAP activity in cytosol of *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours. Values shown are means of six anemone preparations (apart from the 25-31°C value which is a mean of 36 anemones exposed to temperatures in this range for between 24 and 72 hours); error bars show standard deviations.

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0.15 $\mu\text{M}/\text{mg}$ protein respectively. After 48 hours incubation at 35°C cytosolic FRAP values were the highest seen during the entire experimental period at $0.62 \pm 0.11 \mu\text{M}/\text{mg}$ protein, this represents an increase of 170% from values detected in anemones incubated at between 25 and 31°C.

FRAP values per anemone ranged from 0.26 to 0.49 μM (mean 0.35 ± 0.22) during the entire course of the experiment. No obvious trend in response to the temperature regime applied was evident (figure 6.10).

Citrulline formation and FRAP values

Similar trends in protein normalised citrulline formation ($\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$) and FRAP values ($\text{nM}/\mu\text{g protein}$) were apparent over the experimental period (figure 6.11). Values of each parameter were little changed at temperatures between 25 and 31°C, but increases in both parameters were apparent in anemones exposed to temperatures in excess of 31°C. The correlation coefficient derived from plotting citrulline formation rates ($\text{pmoles } \mu\text{g protein}^{-1} \text{ min}^{-1}$) against FRAP values ($\text{nM}/\mu\text{g protein}$) for the entire experimental period was 0.61.

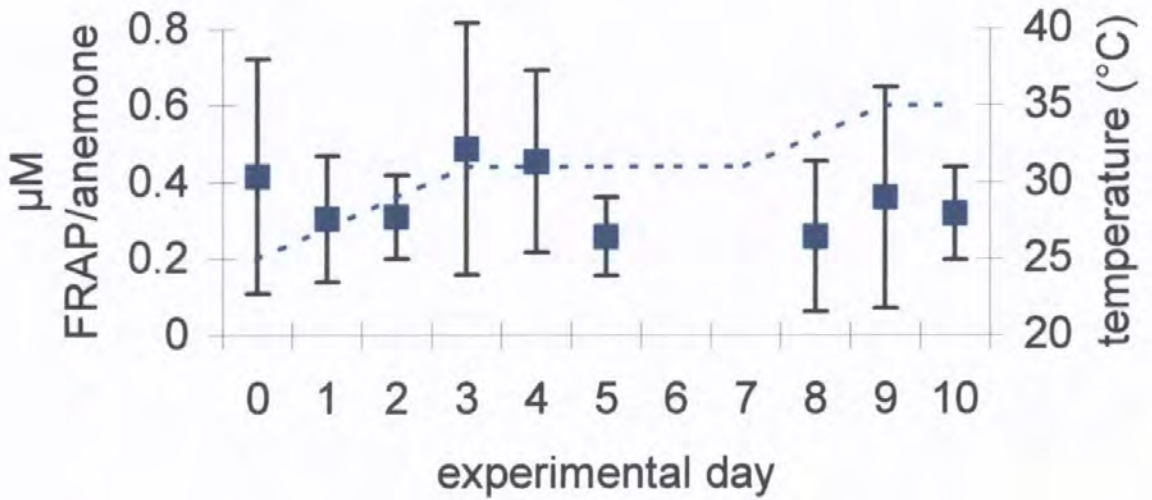


Figure 6.10. FRAP activity per anemone determined from cytosolic preparations of whole *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

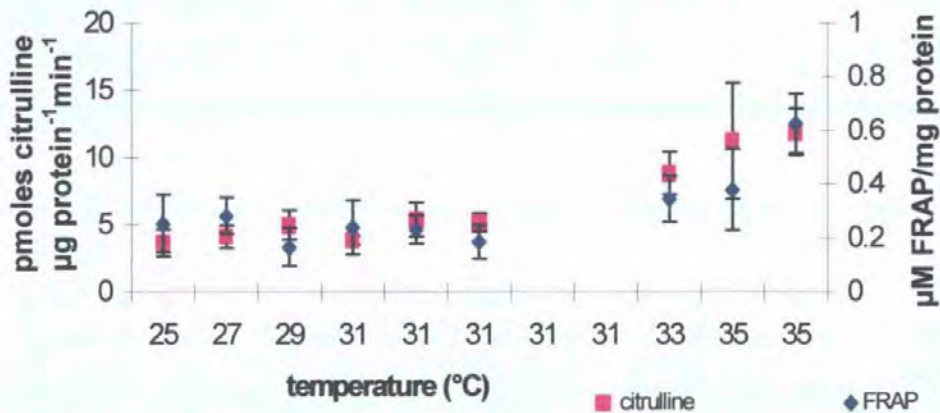


Figure 6.11. Rate of citrulline formation and FRAP activity in cytosolic preparations of whole *A. pallida* incubated at temperatures of between 25 and 35°C for between 24 and 72 hours. Values shown are means of six anemones per treatment, citrulline formation was determined in triplicate; error bars show standard deviations.

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Protein

Considerable variations in protein values were detected during the course of the experiment (figure 6.12) both between groups of anemones (shown by mean values of each experimental day; range from 0.51 ± 0.16 to 1.92 ± 0.43 mg/anemone) and within groups of anemones (shown by bars that indicate standard deviations; maximum range of protein contents within an experimental group 1.06-2.90 (n=6)). Although not statistically significantly different from baseline values, anemones exposed to 29°C for 24 hours, or 31°C for 24 or 48 hours had protein contents each a little under 30% higher than baseline (from anemones maintained at 25°C) values. The lowest protein values were documented in anemones incubated at temperatures above 31°C. Using mean protein levels calculated from anemones maintained at 25°C (experimental day 0) as a baseline, incubation of anemones at temperatures above 31°C resulted in the following reduction in protein levels: 52.5% (33°C, 24 hours), 31.2% (35°C, 24 hours) and 65.9% (35°C, 48 hours).

Zooxanthellae density

Zooxanthellae densities expressed both per anemone and per unit protein (figure 6.13 and 6.14 respectively) showed much variation both within and between experimental days. Anemones maintained at 25°C for 48 hours had zooxanthellae densities of $3.8 \times 10^6 \pm 1.3 \times 10^6$ per anemone; these values were considered background values and other experimental values were compared to these. Anemones exposed to 31°C for 72 hours and those exposed to all temperatures >31°C had lower numbers of zooxanthellae per animal than background anemones. Anemones incubated at 35°C were visibly paler than control anemones. The lowest zooxanthellae densities, ($4.41 \times 10^5 \pm 1.79 \times 10^5$ zooxanthellae per anemone) were recorded in anemones exposed to 35°C for 48 hours in addition to all preceding

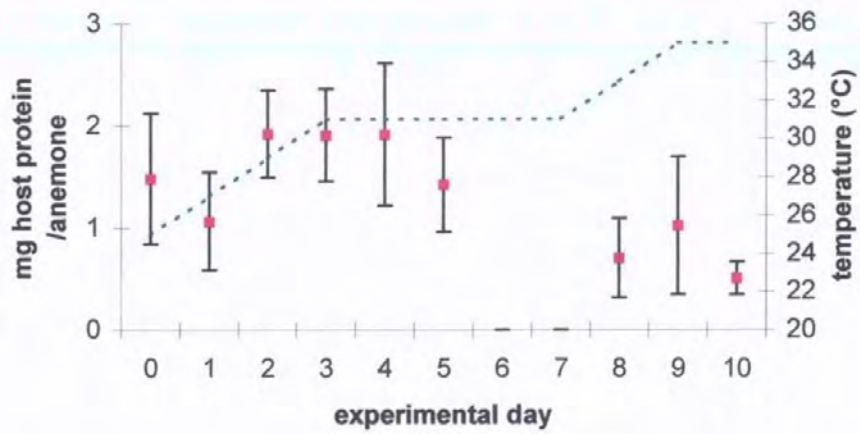


Figure 6.12. Protein contents of *A. pallida* incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

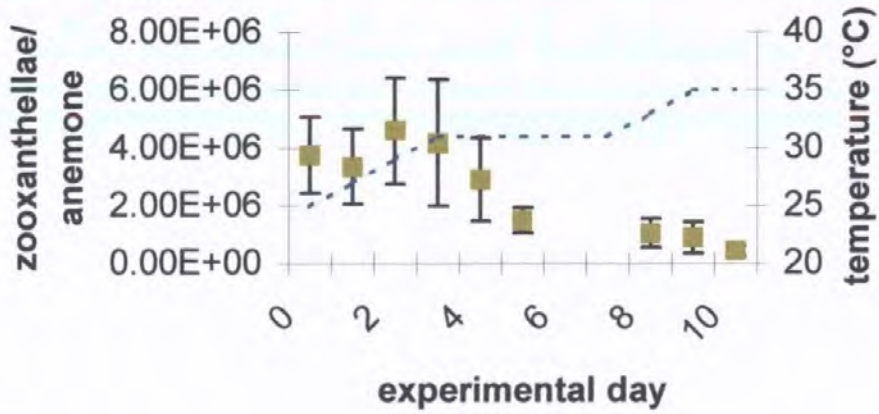


Figure 6.13. Zooxanthellae per anemone incubated at temperatures between 25 and 35°C for between 24 and 72 hours (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

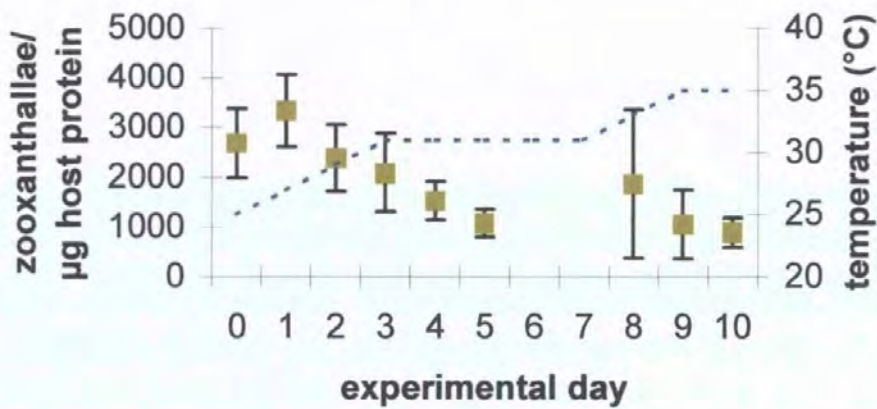


Figure 6.14. Zooxanthellae per µg host protein in anemones incubated at temperatures between 25 and 35°C (applied thermal regime indicated by dotted line). Values shown are means of six anemones per experimental day; error bars show standard deviations.

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temperatures. From start to the finish of the experimental period, zooxanthellae densities in anemones in this experimental group reduced by 88.3%.

Chlorophyll a and c₂ concentration

Chlorophyll a+c₂ contents of zooxanthellae were little changed in anemones exposed to temperatures between 25 and 29°C (mean value $1.2 \times 10^{-5} \pm 1.8 \times 10^{-6}$ µg/ zooxanthellae) (figure 6.15). Increased chlorophyll a+c₂ per zooxanthellae was noted in cells from anemones incubated at both 31°C for 72 hours and 35°C for 24 hours ($2.71 \times 10^{-5} \pm 9.21 \times 10^{-6}$ µg/ zooxanthellae and $2.4 \times 10^{-5} \pm 1.06 \times 10^{-5}$ µg/ zooxanthellae respectively). No clear pattern of change was shown in the total chlorophyll a and c₂ content of anemones exposed to temperatures between 25 and 31°C (figure 6.16). Chlorophyll a + c₂ contents per anemone were considerably reduced in experimental animals exposed to 35°C for either 24 or 48 hours (16.7 and 3.0 µg/anemone respectively). Over the course of the experimental duration the ratio of chlorophyll a: c₂ per zooxanthellae ranged between 3.8 and 5.4 (figure 6.17).

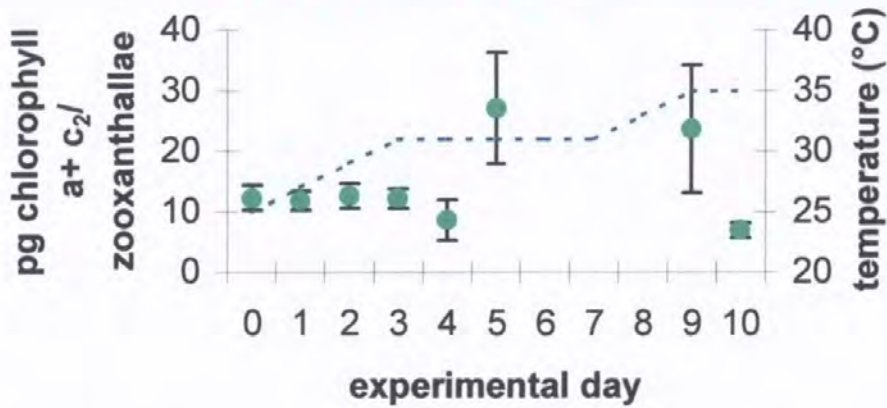


Figure 6.15. Chlorophyll a+c₂ content of zooxanthellae cells from *A. pallida* incubated at between 25 and 35°C (thermal regime indicated by dotted line). Values shown are means of zooxanthellae isolated from six anemones per experimental day; error bars show standard deviations.

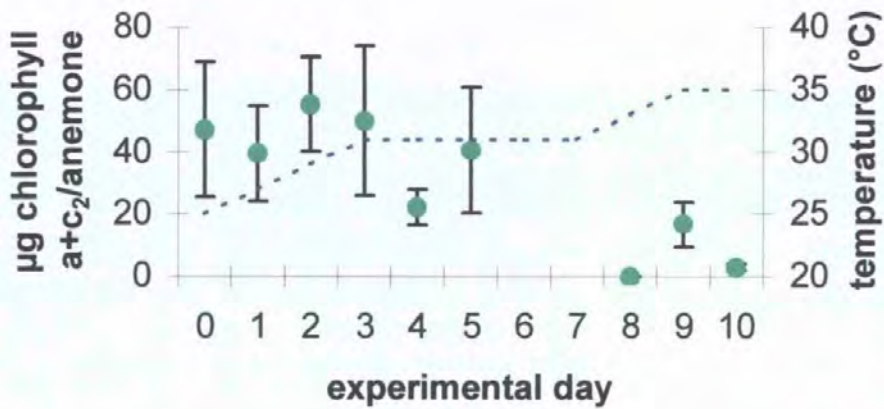


Figure 6.16. Chlorophyll a+c₂ content of *A. pallida* incubated at between 25 and 35°C (thermal regime indicated by dotted line). Values shown are means of zooxanthellae isolated from six anemones per experimental day; error bars show standard deviations.

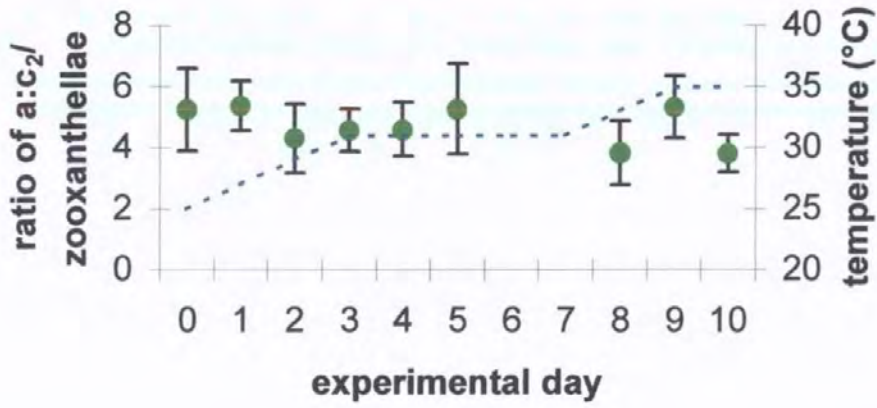


Figure 6.17. Ratio of chlorophyll a+c₂ in zooxanthellae cells from *A. pallida* incubated at between 25 and 35°C (thermal regime indicated by dotted line). Values shown were calculated from mean chlorophyll a and c₂ contents of zooxanthellae isolated from six anemones per experimental day; error bars show standard deviations.

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Physiological data

Total diameter and oral disc diameter data from individual anemones were combined within each treatment group at each time point to give mean values which were plotted against time (figures 6.18 and 6.19). Variations were evident in both the total and oral disc diameter in both control anemones and heat exposed anemones. Total diameters ranged from 9.7 to 20.8 mm in control anemones and from 7.6 to 17.5 mm in anemones exposed to the experimental thermal regime. The smallest diameter recorded was in anemones exposed to 35°C for 48 hours. Regression of total anemone diameters against experimental temperatures gave an R^2 value of 0.1052. Oral disc diameters of control and heat exposed anemones varied between 2.6-4.8 mm and 4.2-6.2 mm respectively over the course of the experiment; no clear pattern of change was apparent in the oral disc diameters of these anemones (figure 6.19).

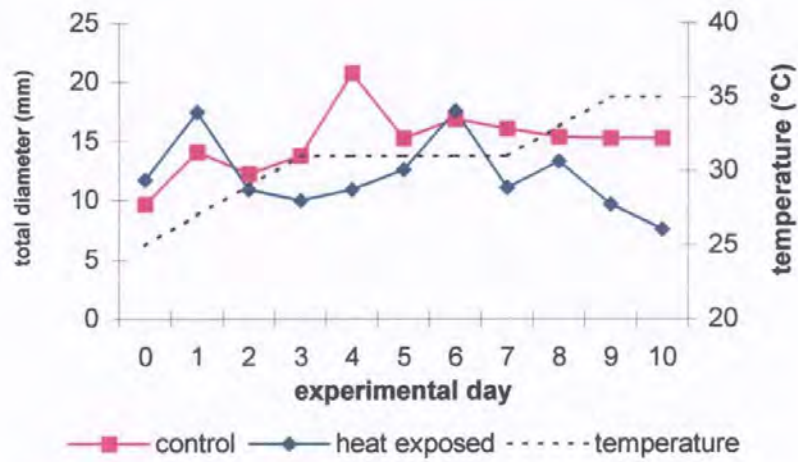


Figure 6.18. Total diameter of *A. pallida* incubated at between 25 and 35°C (thermal regime indicated by dotted line). Values shown are means from six anemones.

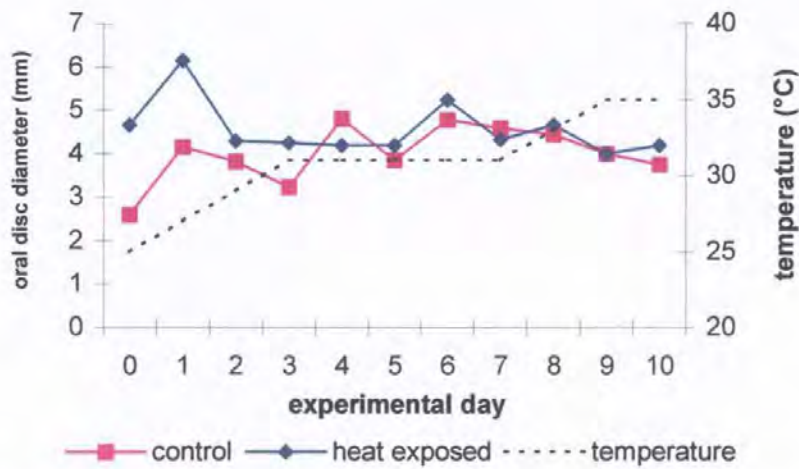


Figure 6.19. Oral disc diameter of *A. pallida* incubated at between 25 and 35°C (thermal regime indicated by dotted line). Values shown are means from six anemones.

6.4 Discussion

Exposure of *A. pallida* to temperatures $\geq 33^{\circ}\text{C}$ over both short (a few hours) and longer terms (over several days) resulted in elevations of NOS activity relative to controls. Bleaching, characterised by marked declines in zooxanthellae densities, was noted primarily in anemones exposed to 31°C for 48 hours. Protein normalised NOS activity levels and FRAP values were similar in anemones exposed to temperatures between 25 and 31°C , but were notably increased in anemones exposed to temperatures of 33 or 35°C . Protein normalised NOS activity levels and FRAP values were similar in anemones exposed to temperatures between 25 and 31°C , but were notably increased in anemones exposed to temperatures of 33 or 35°C . It is important to keep in mind, however, that levels of cytosolic protein content declined dramatically during the course of these experimental treatments. As a result, the overall NOS activity levels and antioxidant capacity per animal were maintained in anemones exposed to temperatures 33°C despite the occurrence of dramatic reductions in cytosolic protein contents in these animals.

Responses to short-term exposure to elevated temperature

Results of an initial experiment indicated lower levels of NOS activity in single anemones exposed to 33 or 17°C for two hours relative to a control anemone maintained at 25°C (Morrall *et al.*, 1998 (appendix 1)). Lower rates of NOS activity were not observed in subsequent experiments that exposed multiple anemones to elevated temperatures. It is likely that the lower levels of NOS activity observed in single anemones exposed to temperatures of 33 or 17°C reflect inter-anemone variance (which was unappreciated at the time) rather than thermally induced changes in levels of enzyme activity. Results of a series of experiments that exposed multiple anemones to 33°C showed levels of NOS activity to

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be consistently increased relative to levels of NOS activity in anemones maintained at 25°C. Levels of NOS activity in control anemones varied between experiments. Possible reasons for variance in basal levels of NOS activity have been discussed previously (chapter three discussion). The specific cause of the increase in NOS activity observed in anemones subject to short-term heat shock is not known at this time. A number of components of the intracellular biochemical environment (such as substrate and/or cofactors or number of potentially inhibitory compounds) may be influenced by ambient temperature and therefore might account for the observed increase in NOS activity per unit protein. Without knowledge of the roles played by NO in *A. pallida*, it is not possible to speculate on the significance of the increased NO production resulting from the observed elevated NOS activity levels. Further work is recommended to elucidate the reasons for, the significance of, and the potential role of increased NO production observed in whole anemones and cytosolic preparations of *A. pallida* in response to elevated temperatures.

Background variance in experimental parameters

Variation was apparent between anemones in each of the assessed parameters (NOS activity levels, antioxidant capacity, zooxanthellae densities, chlorophyll a and c₂ contents and protein contents), indicating that individual *A. pallida* have distinct biochemical and physiological profiles. These distinct profiles are likely to reflect the heterogeneity of the natural habitat and the particular biological history of each individual. A number of suggestions for the observed inter-individual levels of NOS activity are detailed in chapter three: the key sources of NOS activity variance identified in this chapter are applicable to the variance observed in other assay parameters. Determining the reasons for and the significance of the observed variance was beyond the scope of this study, but appreciation of the inherent levels of each parameter is an essential starting point for further studies.

Total antioxidant (FRAP) values

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FRAP values were found to range between 0.11 and 0.36 $\mu\text{M}/\text{mg}$ host protein indicating that protein normalised, combined antioxidant capacity of the non-enzymatic defences of *A. pallida* cytosolic preparations varies between individuals. This is the first study to report FRAP values of a cnidarian preparation. All previously reported FRAP values are expressed as μM per volume (Benzie and Strain, 1999; Benzie and Strain, 1997) in biological fluids for which the total protein content is assumed to remain relatively constant. On a per animal basis (values extrapolated from the volume and weight of each individual anemone prior to dilution and homogenisation) FRAP values varied widely. Inter-individual variations in antioxidant capacity will likely reflect differences in the biomass of experimental animals, which, for cnidarian species can be extremely hard to standardise.

Chlorophyll a and c_2 content and ratios

Zooxanthellae cells isolated from *A. pallida* were found to contain a wide range of chlorophyll a and c_2 contents, indicating that the photosynthetic capacity of these cells is highly variable. Chlorophyll a contents of zooxanthellae determined in these studies were higher, (minimum reported value 5.15 pg/zooxanthellae) than previously reported levels (approximately 1.5-4.55 pg chlorophyll a/zooxanthellae) in zooxanthellae from *A. pallida* and *A. pulchella* (Cook *et al.*, 1988; Dykens and Shick, 1982; Muller-Parker, 1987; Lesser, 1996). Chlorophyll c_2 contents determined in this study were also higher (minimum 1.46 pg/zooxanthellae) than a previously reported value (0.4 pg/zooxanthellae) (Muller-Parker, 1984). Pigment levels of zooxanthellae have previously been shown to vary in anemones from habitats with different irradiance levels and higher chlorophyll a contents have been reported in *A. pulchella* from a shaded mangrove lagoon relative to those from a sunlit reef flat (Muller-Parker, 1987). Irradiance levels reaching the habitat from where *A. pallida* was collected (i.e. submerged roots beneath the mangrove canopy) were not quantified in this study but it is probable that levels are substantially lower than those reaching anemones

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inhabiting reef environments or those maintained in incubators. The high chlorophyll contents detected in zooxanthellae from anemones collected from Walsingham Pond thus may reflect photo-adaptation to the relatively low ambient light levels of this habitat. Freshly collected anemones have been previously noted to have higher chlorophyll contents than laboratory maintained anemones (Cook *et al.*, 1988) and the anemones collected for this work were noted to be particularly dark in colour indicating high concentrations of photosynthetic pigment. Seasonal cycles of chlorophyll contents have been reported for *Anthopleura elegantissima*, with chlorophyll contents noted to vary inversely with mean daily solar radiation (Dyken and Shick, 1984). A relatively small amount of variation in the ratio of chlorophyll a:c₂ was apparent in the assayed anemones, ranging between 3.05 and 4.22. A similar ratio (3.78) has previously been reported for *A. pulchella* collected from a shaded mangrove habitat in autumn (Muller-Parker, 1987). Light intensities were not standardised during the experimental period resulting in fluctuations in ambient conditions of experimental animals. This was an oversight of the experimental design; future studies should standardise levels of illumination and illumination periods to reflect natural conditions.

Protein contents

The protein contents determined for *A. pallida* in this study (0.27-1.56 mg/anemone) are below or at the lower end of the range of previously reported values for this species and for *A. pulchella* (0.89 to 13.4 mg/anemone) (Cook *et al.*, 1988; Muller-Parker, 1984; Muller-Parker, 1987; Ferrier, 1992; Muller-Parker, 1985).

Zooxanthellae densities

Zooxanthellae densities per anemone determined in this study (mean $2.19 \times 10^6 \pm 1.12 \times 10^6$ zooxanthellae per anemone) are similar to those previously reported for *A. pallida* and *A. pulchella* (range $1-4 \times 10^6$ zooxanthellae per anemone) (Cook *et al.*, 1988; Wang and

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Douglas, 1999). A wider range of zooxanthellae per unit protein was obtained in this study (0.57-8.68) than reported in previously studies (range 2.6-3.52 zooxanthellae/mg host protein for *A. pallida* and *A. pulchella*) (Muller-Parker, 1984; Wang and Douglas, 1999, Ferrier, 1992; Muller-Parker, 1987). A number of factors have been reported to influence zooxanthellae densities including feeding frequency (Ferrier, 1992; Muller-Parker, 1985; Cook *et al.*, 1988) and irradiance level (Muller-Parker, 1987). A previous study of *A. pulchella* collected in autumn reports a range zooxanthellae/host protein values of 2.3-3.4 zooxanthellae/mg host protein with a mean of 2.99 (Muller-Parker, 1987), which is slightly lower than the mean value from this study (3.07 zooxanthellae/mg host protein).

These findings illustrate that individual anemones possess distinct profiles of each of the observed biochemical and physiological parameters. Assay of multiple individuals was deemed to be necessary when determining responses of *A. pallida* to a gradually increasing thermal regime.

Responses to exposure to longer term elevated temperatures

This study has shown that subjecting *A. pallida* to a gradually increasing temperature regime, culminating in a 48-hour exposure to 35°C, was sublethal but resulted in morphological changes and reductions in zooxanthellae densities. Both of these changes have been previously documented as cnidarian stress responses (reviewed in section 1.7). It has been previously noted that *Aiptasia pallida* has a fairly broad range of thermal tolerances *in situ* and temperatures of up to 35°C have been used in previous studies with this species (Black *et al.*, 1995). The maximum temperature used in this study (35°C) was 10°C above the ambient temperature at time of collection, and 7.3°C above the reported maximum for the organism's native habitat.

NOS activity levels

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The increases in protein-normalised NOS activity levels seen in anemones exposed to temperatures $>31^{\circ}\text{C}$ are in agreement with the findings of preliminary short-term studies. On a per anemone basis, overall NOS activity levels were either little changed from initial levels or were increased; no clear pattern of change is apparent over the course of the experimental temperature regime. It is of interest to note that despite dramatically lower overall host protein contents detected in anemones exposed to temperatures $\geq 31^{\circ}\text{C}$, overall NOS activity levels showed no decline below initial values. These results are interpreted as showing that NOS activity levels are enhanced or maintained over the course of the applied experimental thermal regime. It is possible that this is a result of *A. pallida* NOS activity being insensitive to the temperature regime. Cnidarian NOS activity has previously been assayed using incubation temperatures of 37°C (Salleo *et al.*, 1996 and Colasanti *et al.*, 1997), therefore the results from this study that show *A. pallida* NOS to remain active at temperatures of 35°C were not unexpected. An alternative explanation for the observed pattern of NOS activity level per anemone is that the organism may regulate enzyme activity in order to maintain NO production. With the realisation that, when expressed on a per animal basis, NOS activity is actually maintained, one can hypothesise that NO, the product of this enzyme, plays such an important role in the physiology of the anemone that, although the stresses of temperature elevation result in a substantial degradation of cytosolic protein, the protein that is NOS retains its activity. It will require more knowledge of the roles played by NO in *A. pallida*, and the ways in which the animal and its symbionts respond to stress, to test this hypothesis. The significance of NOS activity and the resultant NO production during artificially induced thermal bleaching levels while dramatic changes in other physiological parameters are taking place (biomass reduction and breakdown of symbiosis) is unknown at this time but clearly warrants further studies. The production of NO is an energy consuming process; increased NOS activity per unit protein represents an

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energetic cost to the organism. As a consequence, increased levels of NOS activity may result in reduced energetic reserves available for the organism's biological processes such as growth and reproduction.

Antioxidant status

FRAP values per unit protein were similar in anemones exposed to temperatures up to 31°C, but were significantly increased from these levels in anemones exposed to temperatures of $\geq 33^\circ\text{C}$, indicating increased antioxidant capacity per unit protein. The reason for the increased antioxidant capacity per unit protein is unknown but may indicate that the activity of components of the antioxidant system of *A. pallida* were unaffected by the applied thermal regime despite significant declines in host protein contents. Alternatively, the increased antioxidant capacity observed per unit protein may indicate increased production of components of the antioxidant system. This may be a response to increased production of ROS. Oxidative stress (due to increased ROS production) has been demonstrated to be concomitant with acute, sublethal thermal stress in *A. pulchella* (Nii and Muscatine, 1997). Both host and zooxanthellae tissues would be expected to show increased production of active oxygen species due to temperature-enhanced metabolic rates, primarily in the mitochondria (Burdon *et al.*, 1990). Zooxanthellae from *A. pallida* have been shown to have significantly higher concentrations of superoxide radicals and hydrogen peroxide when cultures are exposed to elevated temperatures (31°C) (Lesser, 1996). It has been suggested that protection against active oxygen species is energetically costly both to the animal host (Dyken and Shick, 1984, Shick and Dyken, 1984) and to the zooxanthellae (Lesser and Shick, 1989). Further work is needed to elucidate the energetic costs of increased antioxidant capacity in both *A. pallida* and the associated zooxanthellae. In particular studies need to address how increased antioxidant capacity influences the organisms growth and reproductive potential.

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Despite the increased protein normalised FRAP values reported in anemones exposed to temperatures $\geq 33^{\circ}\text{C}$, no pattern of change in the overall antioxidant status per animal was apparent over the course of the applied thermal regime. Overall antioxidant status was little changed indicating that the capacity of *A. pallida* to mop up free radicals was largely unaffected by exposure to the experimental temperature regime. The absence of a significant decline in overall antioxidant status over a period when host protein levels reduced substantially is notable. A previous study has noted that soluble, non-protein antioxidants are responsible for 80% of the total antioxidant capacity of the temperate sea anemone *Bunodosoma cavernata* (Regoli and Winston, 1998). The composition of antioxidant potential in the anemone *A. pallida* is undocumented at this time but the findings of the study detailed here suggest that the antioxidant status of *A. pallida* is dependent on non-protein components.

A positive association (indicated by a correlation coefficient of 0.61) was found to exist between FRAP values and NOS activity levels per unit protein in *A. pallida* over the entire course of the thermal regime indicating that these parameters vary to some extent together. Both antioxidant capacity and NOS activity levels were found to be increased in *A. pallida* exposed to temperatures $\geq 33^{\circ}\text{C}$. NO exhibits both antioxidant and prooxidant properties depending on the availability and concentration of potential reaction partners such as superoxide and hydrogen peroxide or other reactive oxygen species (Beck *et al.*, 1999). Antioxidant status and NOS activity in *A. pallida*, as well as other species, are thus likely to be linked. Wink *et al.*, (1999) report that protection against oxidative stress requires submicromolar fluxes of NO achievable under normal physiological conditions, while at higher concentrations of NO the effects of reactive nitrogen oxide species may initiate other mechanisms which can lead to tissue injury. The NO donor sodium nitroprusside has been shown to have antioxidant reducing power detectable by the FRAP assay (personal

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observation and T. Galloway, pers. comm.), this suggests that NO could contribute to the antioxidant capacity of *A. pallida*.

In order to understand the significance of the increased levels of NOS activity and increased antioxidant capacity shown to occur concomitantly with bleaching in *A. pallida*, further work is necessary. In particular, the relationship between NO and related redox species, oxidative stress and bleaching needs to be clarified. It is recommended that studies such as these make use of endogenous NO-donor chemicals, arginine analogues and oxidants and antioxidants to manipulate the specific biochemical parameters of interest in the experimental organism. In addition, as the FRAP assay does not measure substantial amounts of serum proteins or low molecular weight SH-group containing antioxidants (Cao and Prior, 1998), use of the ORAC assay (Cao *et al.*, 1993), reported to respond to numerous antioxidants (Cao and Prior, 1998) should be considered in future studies.

Protein

Anemones exposed to temperatures between 25 and 31°C had protein contents of approximately 1-2 mg per animal. These values lie within the previously reported range of protein contents in *A. pallida* and *A. pulchella* (Cook *et al.*, 1988; Muller-Parker, 1984). The highest protein contents reported were in anemones exposed to 29°C for 24 hours or 31°C for 24 or 48 hours. It is possible that the increased protein content of these anemones is a result of increased heat shock protein (hsp) induction, previously noted in this species in response to short term (2 hour) exposure to 29°C (Black *et al.*, 1995). Anemones exposed to temperatures $\geq 33^\circ\text{C}$ had significantly lower protein contents than those exposed to temperatures between 25 and 31°C. Heat shock protein induction has been noted in *A. pallida* exposed to temperatures up to 35°C (Black *et al.*, 1995). Although not apparent from overall protein content results, induction of hsp production in *A. pallida* is likely to have occurred in the animals exposed to temperatures $\geq 33^\circ\text{C}$. Although not noted

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specifically in *A. pallida*, Parsell and Lindquist (1993) report that in some cases, an increase in hsp synthesis is accompanied by a decrease in the production of other non-hsp proteins. This may account for at least some of the decline in protein contents noted in anemones exposed to temperatures $\geq 33^{\circ}\text{C}$. Reduced protein contents have previously been documented in cnidarian host tissues subject to bleaching (Glynn and D'Croz, 1990; Glynn and D'Croz, 1990; Lesser *et al.*, 1990). The mechanism of zooxanthellae density reduction was not noted in this study, but has previously been noted to occur in *A. pulchella* subject to heat shock by loss of zooxanthellae within intact host cells (Gates *et al.*, 1992). Host cell loss occurring in the process of zooxanthellae density reduction may account for some of the noted loss of host protein. However, protein loss lagged behind declines in zooxanthellae densities; significant zooxanthellae density reductions were noted in anemones exposed to 31°C while declines in host protein contents were not noted until anemones were exposed to temperatures of $\geq 33^{\circ}\text{C}$. These results indicate that zooxanthellae density reduction in *A. pallida* may have occurred by a mechanism other than by loss of zooxanthellae within host cells. Reduced anemone protein contents may have resulted from use of proteins to meet the energy requirement of normal, or stress related biological functions. Stress has previously been reported to accelerate protein catabolism in man and animals (Kasschau *et al.*, 1980; Powell *et al.*, 1982) and exposure of *A. pallida* to high concentrations of TBTO has been shown to result in substantial reductions in host protein levels (further detailed in chapter five). In addition to possible stress induced declines in protein contents, it is possible that the lack of feeding, combined with the stress of elevated temperatures applied during the experimental thermal regime may have contributed to the observed decline in protein contents. Significant reductions in protein biomass have been previously shown to occur in *A. pallida* during periods of food deprivation (presumably through catabolism, deamination, and cellular respiration) (Ferrier, 1992). In addition,

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reductions in photosynthate translocation to the host (due to reduced zooxanthellae density, and/or photoinhibition caused by exposure to thermal stress) might exacerbate the host's nutritional deficiency and increase protein catabolism thus contributing to the decline in overall protein content. Further work is required to elucidate the specific cause(s) and significance of the observed reductions in host protein contents in anemones exposed to temperatures $\geq 33^{\circ}\text{C}$.

The significant changes in protein contents of cytosolic preparations observed over the course of long term exposure to elevated temperatures will have altered both citrulline and FRAP assay conditions dramatically. The results of these assays may have been influenced by the variance of assay protein concentrations. As suggested previously (chapter 3), cytosolic protein concentrations used in future assays should be standardised to avoid this possible source of variance.

Zooxanthellae

The zooxanthellae densities reported in anemones at the start of the experiment (approximately 4×10^6 zooxanthellae per anemone and just under 3×10^3 zooxanthellae per μg host protein) are within the range previously reported for *A. pallida* (Ferrier, 1992; Cook *et al.*, 1988) and *A. pulchella* (Muller-Parker, 1984). Bleaching, characterised by reduced numbers of zooxanthellae per anemone, was initially seen in anemones exposed to 31°C for 72 hours. These findings agree with previous reports that have noted that exposure of cnidarians to temperatures of 30°C can result in reductions in zooxanthellae densities (Fitt and Warner, 1995; Lesser *et al.*, 1990). Zooxanthellae numbers declined in terms of densities both per animal and per unit protein in anemones exposed to the thermal regime beyond 72-hour exposure to 31°C . Minimum densities (per animal and per unit protein) were observed in anemones exposed to the entire thermal regime. Zooxanthellae expulsion has been described as being part of a continuous process by which algal densities in the host

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are regulated (Stelle, 1977); it has been reported that symbiotic cnidarians expel less than 0.1% of their standing stock of zooxanthellae per day under normal conditions (Hoegh-Guldberg *et al.*, 1987). The reductions in zooxanthellae densities observed during this experiment exceed what would be expected to occur in these organisms on a routine basis and it is likely that the majority of the density reduction were due to the applied thermal regime. As previously noted, experimental anemones were unfed for the duration of the thermal regime. *A. pallida* previously unfed for durations such as those used in this experiment have been noted to have zooxanthellae densities reduced by about 7% (Ferrier, 1992) and it has been suggested that reduced algal densities may occur as the zooxanthellae are affected by reductions in available host metabolites as they are dependent on host sources of nitrogen and phosphorus (Cook *et al* 1988). In light of these observations, some of the zooxanthellae density reductions shown in this study may be due to nutritional effects. As zooxanthellae densities per anemone were reduced by nearly 90% in animals exposed to the entire thermal regime, it may be that the influence of nutritional status may have contributed to, rather than been the cause of, the observed zooxanthellae density reductions. However it is undocumented at this point how temperature and nutritional status may interact in cnidarian/zooxanthellae symbioses. In addition to influencing zooxanthellae densities on a per animal basis, nutritional status has been reported to affect zooxanthellae numbers per unit protein; increased zooxanthellae per unit protein have been reported in anemones unfed for two weeks relative to those fed daily (Ferrier, 1992). The results of this study show that zooxanthellae densities per unit protein in nutritionally deprived anemones subject to heat stress were reduced, indicating that responses to the thermal exposure may have overridden the influence of nutritional deprivation.

It is well documented that elevated temperatures adversely affect the stability of the symbiotic relationship between cnidarians and zooxanthellae (Glynn, 1990; Hoegh-

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Guldberg and Smith, 1989; Miller *et al.*, 1992; Warner *et al.*, 1990). Temperature strongly affects the rate of carbon fixation, deposition of metabolites by both animal host and its zooxanthellae, and the metabolic balance between host and algal cells (Clark and Jensen, 1982). At elevated temperatures, net oxygen production in anemones and freshly isolated zooxanthellae ceases, probably because of impairment of photosynthetic electron transport at or near the reaction centre (Warner *et al.*, 1996). Thermal effects on biochemical partitioning may have great importance in relation to growth and reproduction of animal hosts of zooxanthellae and the viability of the symbiotic relationship (Clark and Jensen, 1982).

Chlorophyll

Despite dramatic changes in chlorophyll a and c₂ contents per anemone and per zooxanthellae, the ratio of a: c₂ per zooxanthellae was relatively constant over the course of the experimental regime indicating that the changes observed occurred in both pigments. These findings contrast with those of Lesser *et al.*, (1990), who reported significantly decreased ratios of a:c₂ in the zooxanthellae from the zoanthid *Palythoa caribaeorum* exposed to increased temperature (29-33°C compared to controls maintained at 25-27°C). In the absence of significant zooxanthellae loss (up to 24 hours exposure to 31°C) total chlorophyll a and c₂ per zooxanthellae remained stable. A dramatic increase in the combined chlorophyll a and c₂ content per zooxanthellae was apparent in anemones exposed to 31°C for 72 hours; these high levels were largely maintained in anemones exposed to temperatures of 35°C for 24 hours. These results show that the thermally induced bleaching observed in *A. pallida* is characterised by zooxanthellae density reductions, rather than by loss of pigment from intact zooxanthellae. The reason for the dramatic increase in pigment content of the intact zooxanthellae cells is not clear but may illustrate adaptation to the changing microenvironment of the zooxanthellae. Overall

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chlorophyll a and c₂ concentrations per anemone were much reduced in animals exposed to 35°C; these findings are in agreement with those of (Lesser *et al.*, 1990) who reported reduced chlorophyll contents of zooxanthellae of the zoanthid *Palythoa caribaeorum* exposed to increased temperature. Overall reductions in chlorophyll contents indicate that the photosynthetic capacity of these assemblages was reduced following exposure to the experimental thermal regime.

Physiological response

Previous studies have shown exposure of *A. pallida* to temperatures of 27 to 29°C to cause no visible stress (Black *et al.*, 1995); these findings are in agreement with those of this study. Anemones exposed to 35°C were visibly stressed, characterised by whole body contraction and tentacle withdrawal, these findings are also in agreement with those of Black *et al.*, (1995). Responses of this nature are a widely documented general stress response of anemones (previously outlined in chapter 5). The significance and implications of alterations in the degree of expansion/contraction of *A. pallida* have previously been discussed in chapter 5.

Limitations of these studies and recommendations for future work

Comparison of data obtained from the study of background variance and data obtained from experimental day 0 indicates that both the range and means of each of the determined parameters were different on these two occasions. It is possible that this is due to adjustment of *A. pallida* to laboratory maintenance conditions. It is generally accepted that the heat tolerance of organisms is different under laboratory conditions than in the field where they are exposed to more stressors that may act synergistically, as well as in antagonistically (Tsimilli-Michael *et al.*, 1999). This suggests that further work involving field studies of responses to naturally occurring thermal regimes are needed. Exposure of *A. pallida* to thermal stress may have disrupted the symbiotic assemblage by: thermal inactivation of

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photosystem II in the zooxanthellae, disruption of enzymes and the co-ordination of biochemical cycles, physical and enzymatic acceleration of protein and DNA degradation or conformational disruption of both host and zooxanthellae. According to the stress concept proposed by Strasser (1988), any environmental change can be regarded as a stressful event for an organism in the sense that it disturbs the optimality it had achieved through adaptation to its environment.

There is still no consensus on which partner of cnidarian-symbiodinium symbioses is more affected by high temperature (Fitt and Warner, 1995). A number of reports suggest that the animal host is flexible in adapting to thermal conditions while zooxanthellae are more susceptible to, or less adaptable to thermal stress than their cnidarian host (Fitt and Warner, 1995; Clark and Jensen, 1982). Further work is needed to extend knowledge on the thermal sensitivity and responses of each of the symbiotic partners to alterations in thermal regime. Studies of the mechanism(s) of zooxanthellae density reduction were outside the scope of this research but are recommended for future work investigating the relationships between NOS activity, antioxidant status and bleaching.

Salinities of LNSW in experimental dishes were not monitored during exposures of anemones to various thermal regimes. Salinity variations may have occurred between the daily water changes owing to evaporation. The effects of this on the experimental anemones are unknown and it cannot be ruled out that salinity changes contributed to the results obtained.

Conclusions

Exposure of *A. pallida* to temperatures gradually increasing from 25 to 35°C over 10 days induced bleaching characterised by reduced zooxanthellae densities. Increased levels of NOS activity and antioxidant capacity were detected in anemones exposed to this thermal regime. Substantial declines in host protein contents were observed over the experimental

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period. NOS activity levels and antioxidant potential (FRAP) expressed on a per animal basis were maintained despite significant declines in host protein contents shown in anemones exposed to the experimental thermal regime. It is hypothesised that NO, the product of this enzyme, and the antioxidant capacity play such important roles in the physiology of the anemone that, although the stresses of temperature elevation result in a substantial degradation of cytosolic protein and disintegration of the zooxanthellae/host symbiotic relationship, these biological parameters are retained.

CHAPTER SEVEN

OVERVIEW OF

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Overview of Findings and Conclusions

The aim of this project was to investigate the use of nitric oxide synthase (NOS), the enzyme responsible for generation of nitric oxide (NO), as a potential innovative biochemical biomarker for use with cnidarian-zooxanthellae symbiotic associations. A starting point for work aimed at investigating the potential of a novel biomarker is identification and characterisation of the chosen biological parameter. Establishment of foundation knowledge on NOS in *A. pallida* was more time-consuming than was anticipated at the outset of the studies; this was at the expense of more comprehensive investigations of the biomarker potential of the enzyme. Complete validation of the biomarker potential of NOS was not achieved within the time frame of these studies. The preliminary findings documented in this thesis do not support the hypothesis that NOS has potential as a biomarker of contaminant exposure. A more comprehensive knowledge of the roles played by NO in *A. pallida*, in the zooxanthellae symbiotically associated with *A. pallida* and in the symbiotic assemblage as a whole, would assist evaluation of the potential for use of NOS as a biomarker and help to determine if further work in this area is justified. Of particular interest is investigation of the potential signalling role of NO within the cnidarian-zooxanthellae symbiotic assemblage. Elucidation of the role played by NO in specific biological functions, in particular in stress response mechanisms, should be the focus of future studies. Knowledge of the responses of NOS activity to a range of both normally encountered environmental conditions and potentially adverse stimuli should be extended. A comprehensive understanding of the biological and ecological implications of any detected responses is required to validate the biomarker potential of NOS.

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Preliminary findings and limitations of results

Results of studies contained in this thesis document the presence of nitric oxide synthase (E.C. 1.14.13.39 NOS) activity in cnidarian species from shallow marine subtropical environments. Enzyme activity was assayed by measuring the conversion of ^3H L-arginine to ^3H L-citrulline (the citrulline assay). This assay was shown to be sufficiently sensitive for studies of this nature. Post-completion of the work documented in this thesis it was appreciated that the citrulline assay used was not optimised for use with *A. pallida* tissue. A potentially sub-saturating concentration of substrate was provided to the assay incubation and assay incubation times used were beyond the linear period of enzyme activity. These factors, along with use of variable (non-standardized) assay protein concentrations, variable dilution of tissue preparations and use of a hypotonic homogenisation buffer without addition of protease inhibitors, result in the need for caution in interpreting all results generated from the citrulline assay. Thorough testing of the citrulline assay should have been conducted at the outset of these studies to enable modifications to be made to ensure optimal performance and the generation of reliable, accurate results.

NOS activity was demonstrated in the sea anemone *A. pallida*, in the dinoflagellate zooxanthellae *Symbiodinium* spp. isolated from *A. pallida* and in five scleractinian coral species. Considerable variations in basal rates of NOS activity in cytosolic preparations of *A. pallida* were determined over the experimental period. Further research was conducted to investigate the location, role and regulation of the enzyme and the relationship between activity and the physiological status of the organism. The objective of this research was to investigate the potential of NOS as an indicator of organismal changes occurring in response to environmental stress. To this end, a partial characterisation of the enzyme was conducted, enzyme localisation was determined and both enzyme and morphological

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responses to NOS inhibitors, an NO donor, selected contaminants or a thermal regime that induced bleaching were investigated.

Characterisation of NOS

NOS activity of *A. pallida* was shown to be kinetically characterised by an apparent K_m of 132.9 μM for L-arginine and an apparent V_{max} of 17.7 pmoles citrulline $\mu\text{g protein}^{-1} \text{ min}^{-1}$. NOS activity was inhibited by the arginine analogue L-NMA with an apparent K_i of 1014 μM ; complete inhibition was not achieved even at high concentrations of L-NMA. NB: The term apparent is used as a result of the largely unpurified form of the enzyme and use of a non-optimised assay in determination of these kinetic parameters. Results showing that the arginase inhibitors L-valine and L-ornithine did not diminish NOS activity detected by the citrulline assay were interpreted as evidence that citrulline generation was not occurring via the activity of arginase. NOS activity levels were apparently unaffected by exposure to the immune stimulant LPS suggesting that *A. pallida* NOS is a constitutive rather than inducible isoform. These findings suggest that there are similarities between the biochemical characteristics of NOS from *A. pallida* and those reported for previously studied isoforms.

Localisation of NOS

A. pallida NOS was shown to be located in the cytosolic fraction of whole organism preparations. Histochemical localisation of NOS activity by NADPH-diaphorase staining showed the enzyme to be present in the epidermal cells and at the extremities of the mesoglea. This staining pattern is compatible with a broad involvement of NO in both sensory and motor activities of the organism. Further work, i.e. staining tissues after sectioning, to investigate the role of the products of the NOS activity shown by NADPH-d staining of *A. pallida* tissues is recommended. Roles of NO in signalling have been shown in a wide range of vertebrate and invertebrate species. NO is able to diffuse through cell

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membranes and is capable of rapidly eliciting responses. The possibility that NO is involved in the rapid contraction of stalk and tentacles shown by *A. pallida* in response to ambient conditions predicted to be deleterious should be further investigated. Most physiological responses triggered by moderate amounts of NO are mediated by activation of soluble guanylyl cyclase and the subsequent production of cyclic GMP (Beck *et al.*, 1999). It is recommended that the possibility that NO plays a signalling role in *A. pallida* is investigated starting with use of the methods of Elphick *et al.*, (1993) with *A. pallida* tissue, NO donor compounds and assays of levels of the cyclic nucleotides cAMP and cGMP.

Responses to exposure to contaminants, arginine analogues and an NO donor

Levels of NOS activity were not altered in *A. pallida* in any systematic way by exposures to copper, fluoranthene or tributyltin-oxide (TBTO). Oversight of the crucial importance of determining actual exposure concentrations limits the usefulness of these findings. Future work should combine knowledge of actual contaminant concentrations with knowledge of the bioavailability, uptake, accumulation and elimination of contaminants, in order to facilitate a full understanding of the observed biological responses.

Rates of NOS activity in *A. pallida* exposed to copper or TBTO varied from control levels but no clear relationship was shown between activity levels and predicted concentrations. An attempt was made to relate the ecological significance of the measured responses by comparison with other biomarkers of cellular (with use of the lysosomal stability assay) and organismal (morphological status) biomarkers. Interpretation of contaminant responses elicited by *A. pallida* was limited by an inability to cross reference responses with measures of lysosomal stability, a well-validated index of cellular stress. The reason for the failure to visualise lysosomes (which prohibited cross referencing of results) is not clear. Development of preparatory methodologies that enable visualisation of lysosomal structures

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in cnidarian preparations should be a focus of future work. This would facilitate assessment of the potential usefulness of lysosomal stability monitoring in cnidarian species.

The absence of a clear dose response relationship between NOS activity levels and copper, fluoranthene and TBTO in the concentration ranges and time periods tested in these studies is not supportive of the hypothesis that NOS has potential as a sensitive biomarker of exposure to these contaminants. However, the results presented here are those of preliminary studies. Further investigation into the involvement of NO in stress responses of cnidarian-symbiodinium and other species are needed in order to, a) clarify if a role is played by NO in *A. pallida* contaminant responses and, if justified, b) to further test the hypothesis that NO or NOS has potential as a biomarker of contaminant exposure and/or effect. NO has been reported to be an activator of the protein SoxR which regulates genes whose products mediate an array of cellular defences, including resistance to heavy metals (Hidalgo *et al.*, 1997). Studies conducted at the level of gene expression may be valuable in studies aimed at elucidating the involvement of NO in cellular resistance in *A. pallida*.

Morphological responses (as an indication of the health status of the organism) were monitored in *A. pallida* during exposure to copper, fluoranthene or tributyltin-oxide. Contraction of tentacles and body columns was a frequently noted response to each of these exposures. In addition to contraction responses, increased mucus production and disruption of the *A. pallida*-zooxanthellae symbioses resulted from exposure to high concentrations of TBTO or copper. Exposure of whole anemones to arginine analogues resulted in tentacle and body column contraction, however no clear relationship between morphological status and levels of NOS activity was found.

Results of work presented here have shown that exposure to a variety of contaminants and NO related chemicals elicit a common stress response in anemones shown as body and tentacle contraction, and in some cases tentacle withdrawal. Although these studies did not reveal a clear relationship between NOS activity levels and morphological status of *A.*

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pallida, further work is required to investigate if NO is involved in the mechanism by which morphological responses are brought about. The morphological responses elicited by whole *A. pallida* exposed to chemicals known to interfere with NOS activity (i.e. arginine analogues or exogenous NO donors) indicate that these chemicals have a role to play in future investigations aimed at elucidating the role played by NO in this species. Determination of the biological consequences of varying levels of NO may be achieved by manipulating endogenous NO concentrations by blocking synthesis of NO with arginine analogues or enhancing endogenous levels of NO with NO donors. As NOS activity was reduced most markedly by the arginine analogue L-NMA (less enzyme activity inhibition resulted from equivalent treatments with L-NNA and L-NAME), use of L-NMA is suggested in future studies. It is essential to studies designed to manipulate NO levels that the specificity of action of the agent used is determined. This can be achieved by use of the D-enantiomer of the arginine analogue used (i.e. D-NMA in the case of L-NMA), and use of the non-NO releasing NO-donor analogue (i.e. potassium ferrocyanide for sodium nitroprusside). Results of studies of this kind will contribute to understanding the roles played by NO in *A. pallida* and the significance of alterations in rates of NO generation. It may be possible to extrapolate this information to species closely related to *A. pallida* and more broadly, results of studies of this kind will enhance understanding of the significance of NO in lower invertebrate species.

Responses to elevated temperatures

The ecological significance of NOS activity and its relationship to environmental stress was investigated by exposing *A. pallida* to a thermal regime that induced bleaching, perhaps the most comprehensively documented stress responses of any of the invertebrates. Determination of background levels of NOS activity levels, antioxidant capacity, zooxanthellae densities, chlorophyll a and c₂ contents and protein contents in multiple

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anemones newly collected from their natural environment revealed considerable inter-individual variation in each of these parameters. Ratios of biological material to HB were not maintained at a constant level. As a result, it is possible that at least part of the inter-individual variance in parameters such as NOS activity levels and antioxidant capacity were influenced by variations in the concentration of biological material in assays. Protein concentrations in future assays must be standardised.

Exposure of *A. pallida* to temperatures gradually increasing from 25 to 35°C over 10 days induced bleaching characterised by reduced zooxanthellae densities. Anemones exposed to temperatures $\geq 33^\circ\text{C}$ showed substantial reductions in size as a result of stalk and tentacle contractions. These findings suggest that morphological monitoring may have a role to play as an indicator of exposure to extreme temperatures. In order to validate the potential of morphological monitoring in environmental stress response investigations, studies of the morphological responses resulting from exposure to environmentally realistic thermal regimes are needed, along with studies of the biological and ecological significance of these responses. Substantial declines in host protein contents were observed over the experimental period. Despite the dramatic reductions in cytosolic protein contents, higher levels of NOS activity and increased FRAP values were detected in anemones exposed to the upper experimental temperatures. The resilience of NOS activity and antioxidant capacity to temperatures at the upper lethal limit of the species they were isolated from may indicate that fundamental physiological roles are played by these parameters. Further studies are needed to investigate these roles and to determine the significance of NO and antioxidant capacity during all stages of bleaching. The relations between these biochemical parameters should be further investigated, particularly with regard to the possible contribution of NO to antioxidant capacity and/or oxidant load. As past research has implicated NO in alteration of protein function and the initiation of gene expression to protect cells (Beck *et al.*, 1999),

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it is suggested that initial studies focus on these potential functions during the onset and progression of bleaching events.

Recent work provides evidence that indicates that oxidative stress plays a central role in thermally induced coral bleaching (Downs *et al*, 2000). The precise mechanism of bleaching is yet to be elucidated. The FRAP assay enabled detection of altered antioxidant capacity in *A. pallida* that were bleaching as a result of exposure to elevated temperatures. This suggests that antioxidant capacity was sensitive to changing environmental conditions and as such, monitoring this biochemical parameter may prove useful as a biomarker. The biological and ecological relevance of alterations in antioxidant capacity need to be determined in order for the biomarker potential of this parameter to be established. The FRAP assay was found to be a quick and straightforward means of determining the antioxidant potential of cnidarian tissues. The assay was reliant on standard laboratory equipment (i.e. a spectrophotometer) indicating that the assay could be conducted in basically equipped laboratories. Quantification of antioxidant capacity using the FRAP assay, both in cnidarians and possibly in the associated zooxanthellae, is recommended in further studies of the mechanism of action of oxidative stress in coral bleaching events. However, as the FRAP assay does not measure substantial amounts of serum proteins or low molecular weight SH-group containing antioxidants (Cao and Prior, 1998), use of the ORAC assay (Cao *et al.*, 1993) may prove to be as more comprehensive means of determining total antioxidant capacity. Further to studies of the involvement of oxidative stress in bleaching, monitoring the antioxidant capacity of organisms may prove valuable in contaminant response studies. Augmented oxyradical production has been reported in aquatic organisms resulting from anthropogenic pollutant outfalls (Winston and Di Giulio, 1991). The toxicological consequences of this will depend largely upon the organism's antioxidant defences. This indicates that antioxidant capacity is crucial to determining the biological responses of organisms to pollutant toxicity.

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Experimental assays

While use of the citrulline assay was proven to be sufficiently sensitive for the undertaken studies, dependence on this assay as a means of quantifying NOS activity/ NO production may prove to be restrictive owing to its reliance on radiolabeled arginine (use of which requires specialised facilities) and use of a scintillation counter (which is specialised equipment). The greiss assay (as outlined by Tracey, 1992) relies on the detection of nitrate and nitrite as a means of determining NO production using a spectrophotometer and is likely to prove to be a technique that is more widely accessible than the citrulline assay. Assays that are simple and reliant on standard laboratory resources are particularly important to developing nations who may not have access to specialised equipment and materials. Before the greiss assay is viable alternative to the citrulline assay for use with cnidarian tissue preparations, the assay must be optimised for use with these specific biological preparations.

Morphological responses

Contraction responses were observed in *A. pallida* exposed to a variety of ambient conditions suspected to be detrimental to the organism. These results indicate that this response is stress sensitive, an essential characteristic of any biomarker. Quantification of morphological responses to stresses required only basic skill, was quick and was reliant upon (in terms of resources) a simple measuring device, experimental dishes and organisms. These assay attributes indicate that morphological response monitoring could be a useful biomarker of non-specific stress responses in *A. pallida*. Extrapolation of this technique to other cnidarian species is likely to be possible once knowledge of contraction/expansion behaviour exhibited under normal conditions is obtained. Identification and definition of specific stages in responses to adverse conditions would enable quantification of results and would facilitate comparison of observed responses, both between different magnitudes of

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the same treatment and/or exposure periods, and between different treatments. Use of automated time-lapse photography or a continuous recording device, such as a video camera, could enhance studies of this kind by providing a permanent record of the progression of response exhibited. Investigation of the biological and ecological significance of contaminant induced morphological responses is also recommended (i.e. determination of the nutritional impact in host and symbiont of varying degrees and longevities of tentacle and body contraction). This would provide information on the biological implications of the observed responses, which would enhance the ability to interpret results from morphological response studies and enable the ecological relevance of the response to be quantified. The inherent morphological changes shown by *A. pallida* in this study suggests that use of multiple anemones is necessary to prevent natural variations masking responses to adverse stimuli.

Overview of findings

The research documented in this thesis provides a preliminary characterisation of NOS in *A. pallida*. The absence of careful preliminary work to optimise the citrulline assay is regrettable. There is much scope for further biochemical investigations, for example using purified *A. pallida* NOS to determine cofactor requirements and kinetic characteristics in the absence of potentially interfering biochemical substances. The preliminary contaminant response results presented here could be furthered and extended to encompass environmentally realistic sublethal exposures using larger sample numbers and longer exposure periods than were used here. It is recommended that the responses elicited by cnidarian-zooxanthellae symbiotic assemblages in response to exposure to environmentally realistic levels of contaminants or conditions identified as particular threats to subtropical and tropical shallow marine environments should be monitored in future studies. Of importance to further studies of morphological and biochemical responses to contaminant

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exposures is determination of contaminant body burdens. This would enable the relationship between contaminant load and observed effects to be elucidated. In addition, it is essential that the ecological relevance of the biological responses to contaminants or environmental conditions suspected to be detrimental is determined. The studies documented here focused largely on the biochemical responses of only part of the symbiotic assemblage to contaminants, chemicals and elevated temperatures. It is recommended that future experiments be designed to simultaneously monitor responses in both host and symbiont. Small sample numbers were used in order to preserve natural stocks of *A. pallida*. The establishment of substantial stocks of laboratory-cultured anemones would allow use of larger sample numbers in future studies. This is likely to reduce the background inter-anemone variance of biochemical and physiological parameters observed (owing to the standardised environmental conditions under which the anemones are maintained) and would enable use of a wider range of statistical tests on the experimental results obtained.

Conclusions

Shallow tropical and subtropical marine environments are highly ecologically and economically valuable but are threatened by a range of natural and anthropogenic threats. Knowledge of the biological consequences of these threats is essential to environmental risk assessment, environmental protection and sound environmental management. Biomarkers are valuable tools in ecological risk assessment but currently few biomarkers have been validated for use in tropical and subtropical marine environments. Further work aimed at developing robust biomarkers for use in environmental risk assessment in shallow tropical and subtropical environments must be conducted to address this current knowledge deficit. To ensure all nations with tropical and subtropical marine environments can utilise the developed tools (including the developing nations that constitute the majority of countries that border tropical and subtropical marine environments), biomarkers developed for these

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regions must be inexpensive and use assay methodologies that rely on low technology equipment. As the key structural framework of the shallow tropical marine environment and owing to their proven sensitivity to a wide range of environmental perturbations, cnidarian-zooxanthellae symbioses should be utilised in these studies.

Development of biomarkers appropriate for use with key species in shallow marine environments of the tropics and subtropics will assist environmental risk assessment in these areas but action by all members of the international community is urgently required to reduce the deleterious impacts that threaten these environments. The prime threats to coral reefs: eutrophication, over-fishing and climate change (Roberts *et al.*, 2002), are at least partly anthropogenically caused. In order that they be preserved, alongside efforts to develop biomarkers suitable for use in shallow tropical and subtropical marine environments, efforts must be made to mitigate the threats posed to these productive, biodiverse, spectacular environments.

APPENDICES

Appendix 1

Published manuscripts

1. Morrall, C.E., Trapido-Rosenthal, H.G., Knap, A.H. and Depledge, M.H. Development of nitric oxide and nitric oxide synthase as ecotoxicological biomarkers in the tropical marine environment. *Marine Environmental Research*, Vol. 46, (1-5): 429-432, 1998.
2. Morrall, C.E., Galloway, T.S., Trapido-Rosenthal, H.G. and Depledge, M.H. Characterisation of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Journal of Comparative Biochemistry and Physiology*. 125: 483-491, 2000.
3. Trapido-Rosenthal, H.G., Sharp, K.H. Morrall, C.E. and Galloway, T.S. Nitric oxide and cnidarian-dinoflagellate symbioses: pieces of a puzzle. *American Zoologist*. 41: 111-121, 2001.



Development of Nitric Oxide and Nitric Oxide Synthase as Ecotoxicological Biomarkers in the Tropical Marine Environment

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ABSTRACT

The enzyme nitric oxide synthase (NOS) catalyses reactions that result in the generation of nitric oxide (NO), a multipotent signal molecule. We have begun to characterise the activity of NOS found in the host tissue of the symbiotic tropical/sub-tropical anemone Aiptasia pallida, and have monitored changes in the activity of this enzyme under different environmental conditions. Our characterisation studies indicate that the NOS activity from Aiptasia is similar to activities from other organisms in terms of cellular localisation, cofactor requirements, and inhibition profiles. Our environmental experiments indicate that: (1) the incubation of whole animals at temperatures representative of the highest and lowest extremes to which the Bermuda population would be exposed results in an NOS activity reduction relative to animals maintained at the control temperature of 25°C; and (2) treatment with copper produces variable changes in NOS activity relative to untreated controls. © 1998 Elsevier Science Ltd. All rights reserved

It has recently been recognised that nitric oxide (NO) is a molecule of fundamental biological significance, being involved in cellular and organismal responses to a variety of stresses. NO differs from many molecules used in chemical signalling in several fundamental aspects. Since it passes through cellular membranes, it cannot be stored in vesicles and exocytosed on demand. This necessitates generation on an as-needed basis.

Nitric oxide synthase (NOS: EC 1.14.13.39) is responsible for catalysing the conversion of L-arginine to L-citrulline and NO. A goal of our laboratory is to develop assays that will be of value in monitoring the responses of tropical marine ecosystems to environmental stresses. In pursuit of that goal, the specific aims of the work described herein are to (1) characterise the enzyme NOS in the sea anemone *Aiptasia pallida* and (2) investigate the responses of the NOS activities in whole organism exposed to either thermal stress or exposure to copper ions.

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The symbiotic anemone *Aiptasia pallida* (Verill) was used for all experiments. Experimental organisms were taken from a clonal population of anemones, collected from mangrove roots in Walsingham pond, Bermuda. Anemones were maintained in 100-ml dishes containing low-nutrient surface seawater (Insw), at 25°C on a 12 h light/12 h dark schedule. Stocks were fed twice weekly with *Artemia spp.* nauplii. [^3H]Arginine (40 Ci mM $^{-1}$; 1 Ci = 37 Gbq) was obtained from New England Nuclear. All other chemicals were reagent grade, and were obtained from Sigma, USA. Individual *A. pallida* were homogenised in a Wheaton glass homogeniser in 2 ml homogenisation buffer (50 mM HEPES/1 mM EDTA, pH 7.4); such homogenisation procedures leaves zooxanthellae intact (Muscattine *et al.*, 1991; Billinghamurst *et al.*, 1998). Homogenates were spun at 14 000 g for 5 min to separate soluble host material from zooxanthellae and insoluble host tissue. NOS activities were then determined in homogenates of host tissue by monitoring the conversion of 1 μM arginine to citrulline according to the method described by Bredt and Snyder (1989). Activity was expressed as picomoles of citrulline produced per μg protein per min incubation; protein concentrations were determined by means of the dye-binding assay of Bradford (1976) using bovine serum albumin standards. Results are combined data from three repeat experiments; except where otherwise stated, three individuals were used for each treatment. Location of NOS activity was examined by comparison of activity in the supernatant of host homogenate with activity in a sample of homogenate that had not been subjected to centrifugation. Dependence of *A. pallida* NOS activity on Ca^{++} and calmodulin was investigated by dividing the supernatant of the homogenate from a single anemone into three aliquots, which were then incubated as follows: (1) standard composition, (2) no exogenous Ca^{++} , (3) no exogenous calmodulin. Effects of inhibitors were investigated by including potential inhibiting compounds at a concentration of 10 mM. Enzyme kinetics were investigated over a range of arginine concentrations (2×10^{-6} to 2×10^{-4} M). The effect of thermally stressing animals on NOS activity was investigated by incubating anemones at either 17 or 33°C for 120 min, prior to homogenisation and assay; results were compared with those obtained from control animals maintained at 25°C. Effects of copper were examined by incubating anemones in Insw that contained $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ at concentrations of from 1 to 4 $\mu\text{g l}^{-1}$; incubations were for 24 h, at 25°C, with continuous gentle water agitation.

Aiptasia NOS activity appears to be cytoplasmic in location; whole homogenate generated 3.1 ± 0.4 picomoles citrulline μg^{-1} protein, while the supernatant of that homogenate generated 3.4 ± 0.4 picomoles citrulline μg^{-1} protein. The activity, required both Ca^{++} and calmodulin for maximal activity: in the absence of exogenous Ca^{++} activity was reduced by 54%, whereas in the absence of calmodulin, it was reduced by 27%. In the presence of 10 mM *N*-nitro-L-arginine methylester, activity was reduced to 66% of control values; activity in the presence of 10 mM L-valine was indistinguishable from activity in the control condition. The kinetics of the conversion of arginine to citrulline were characterised by a K_M of 110 μM , and a V_{max} of 13.0 pmoles mg^{-1} protein min^{-1} . Incubation at both 33 and 17°C for 2 h resulted in a reduction of enzyme activity relative to control 25°C incubations (Fig. 1). Figure 2 indicates that we have found no clear dose-dependent response of NOS activity to copper ions. Both increases and decreases in activity can be seen at different concentrations.

The evidence we have accumulated to date suggests that *Aiptasia* NOS activity bears similarities to NOSs that have been characterized from other organisms. It is a cytoplasmic, rather than a membrane-bound activity; the activity appears to be optimal in

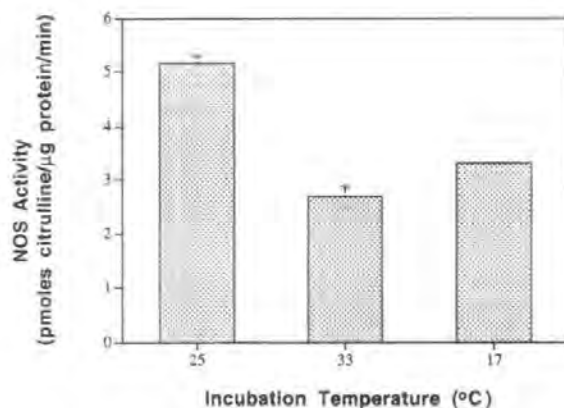


Fig. 1. Effect of temperature on *Aiptasia* NOS activity. Anemones were incubated at the indicated temperatures for 2 h prior to homogenization and assay. Means and standard errors of means of triplicate assays are indicated; in the absence error bars, standard errors were less than 5% of the mean value.

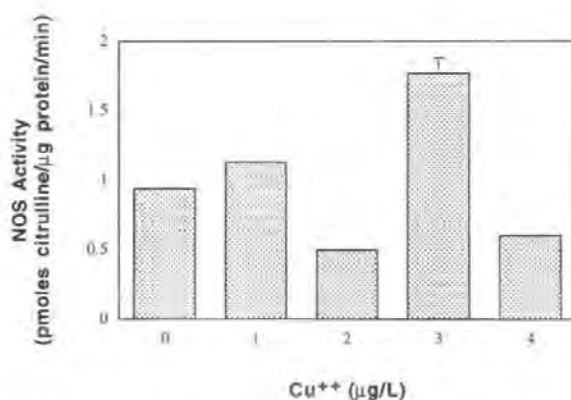


Fig. 2. NOS activity in *Aiptasia* cytosol following incubation of whole anemones in the indicated concentrations of CuCl_2 for 24 h. Means and standard errors of means of triplicate assays are indicated; in the absence error bars, standard errors were less than 5% of the mean value.

the presence of calcium ions and calmodulin; and the activity is subject to inhibition by the NOS inhibitor nitroarginine, but insensitive to inhibition by the arginase inhibitor valine. Our findings indicate NOS activity is significantly down-regulated by subjecting *Aiptasia* to temperature stress. Curiously, both increased or decreased temperatures result in a decrease in NOS activity. The decline in activity in response to temperature stress is relatively rapid—30 min of treatment has the same effect as does 120 min (data not shown). The ambiguous results we obtained upon dosing organisms with copper remain unexplained at this point. Previous work on uptake of copper from seawater by the anemone *Anemonia viridis* indicated the anemones did not take up metal in proportion to external concentrations (Harland and Nganro, 1990). Copper ions may also interact in a

number of ways with NO or one or more of its reaction products, or with NOS itself; one or more of these interactions could be rendering our experimental procedure inappropriate.

Our work to date suggests that assaying NOS activity in *Aiptasis pallida* may be a valuable means of monitoring the response of tropical marine organisms to some, but not all, classes of environmentally encountered stress. We now need to elucidate the significance of changes in the enzymatic activity in terms of the mechanism of stress responses and subsequent alterations in organismal fitness.

ACKNOWLEDGEMENTS

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REFERENCES

- Billinghamurst, Z., Douglas, A. and Trapido-Rosenthal, H. (1998) *Proc. 8th Intl. Coral Reef Symp.* (in press).
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Bredt, D. S. and Snyder, S. H. (1989) *Proc. Natl. Acad. Sci.* **86**, 9030–9033.
- Harland, A. D. and Nganro, N. R. (1990) *Marine Biology* **104**, 297–301.
- Muscatine, L., Grossman, D. and Doiño, J. (1991) *Mar. Ecol. Prog. Ser.* **77**, 233–243.



Characterisation of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*

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Abstract

The presence of nitric oxide synthase (EC 1.14.23 NOS) activity is demonstrated in the tropical marine cnidarian *Aiptasia pallida* (Verrill). Enzyme activity was assayed by measuring the conversion of [³H]arginine to [³H]citrulline. Optimal NOS activity was found to require NADPH. Activity was inhibited by the competitive NOS inhibitor N^G-methyl-L-arginine (L-NMA), but not the arginase inhibitors L-valine and L-ornithine. NOS activity was predominantly cytosolic, and was characterised by a K_m for arginine of 19.05 μ M and a V_{max} of 2.96 pmol/min per μ g protein. Histochemical localisation of NOS activity using NADPH diaphorase staining showed the enzyme to be predominantly present in the epidermal cells and at the extremities of the mesoglea. These results provide a preliminary biochemical characterisation and histochemical localisation of NOS activity in *A. pallida*, an ecologically important sentinel species in tropical marine ecosystems. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Citrulline assay; Cnidaria; Coral; Invertebrate; NADPH diaphorase; Nitric oxide synthase; Nitric oxide; Sea anemone

1. Introduction

Nitric oxide (NO) is a multi-functional messenger that has been identified in the majority of animal phyla, in bacteria and in a variety of plant species. NO has been implicated in many fundamental cellular processes (Nathan and Xie, 1994) and is generated by a family of nitric oxide synthase (NOS) isozymes which differ in their mode of expression, primary sequence and calcium dependency (Moncada and Higgs, 1993). These different isoforms have been characterised in mammalian species. A constitutive Ca²⁺/calmod-

ulin-dependent cytosolic isoform, nNOS, has been located in neuronal tissues, and subsequently also in skeletal muscle, epithelial cells and other tissues (Griffiths and Stuehr, 1995). The Ca²⁺-dependent membrane-associated form, eNOS, is found in vascular endothelial cells and is activated by Ca²⁺ influx, agonists such as bradykinin and by shear stress (Janssens et al., 1992). A third Ca²⁺-independent isoform is induced in response to bacterial lipopolysaccharide (LPS) and a variety of cytokines, notably interleukin-1 and interferon- γ (Stuehr, 1997). It is found primarily in macrophages, but is also induced in other tissues e.g. endothelial cells and hepatocytes. Constitutive expression of iNOS may also occur in tissues such as lung epithelium (Nathan and Xie, 1994). Whilst calmodulin is permanently bound to iNOS, it is

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insensitive to fluctuations in cellular Ca^{2+} . The main isozymes differ in size from 130 to 160 kD (Crane et al., 1997) but all share the same general three component structure and mode of action (Stuehr and Griffiths, 1992).

NO exerts many of its effects by activating the enzyme soluble guanylyl cyclase to generate the second messenger cyclic GMP (cGMP) (Garthwaite, 1991). The ancient origins of this signaling pathway in evolution are illustrated by the near ubiquitous identification of NO-mediated functions across the animal phyla. These include cnidarians (Colasanti et al., 1995), nematodes (Bascal et al., 1995), annelids (Leake and Moroz, 1996), molluscs (Conte and Ottaviani, 1995), arthropods (Radomski et al., 1991) and vertebrates. NOS activity has been characterised in the bacterial species *Nocardia* (Chen and Rosazza, 1994) and has recently been shown to play a role in the defence against pathogens in plants (Delledonne et al., 1998).

Symbiotic cnidarians such as the anemone *A. pallida* (Verrill) (= *A. tagetes* sensu (Verrill, 1900) and a variety of coral species largely dominate the marine ecosystems of the tropics and subtropics. *A. pallida* and its Pacific congener *A. pulchella* are widely distributed and can be easily collected and maintained in the laboratory. These attributes, along with the organisms close taxonomic proximity to many coral species have made it the organism chosen for much past and current research.

Previous reports have tentatively identified NOS activity in the cnidarian species *A. pallida* (Elofsson et al., 1993) and *A. diaphana* (Salleo et al., 1996) using histochemical staining but the exact location and biochemical characteristics of the enzyme have not been previously reported. Here, the presence of NOS activity in *A. pallida* is confirmed via the measurement of [^3H]citrulline. The substrate and cofactor requirements of the enzyme are investigated and the response to inhibitors is documented. The NADPH diaphorase staining technique is used to localise NOS activity to cells in the epithelial layer and those occurring at the edges of the mesoglea.

2. Materials and methods

2.1. Materials

[^3H]-L-arginine (40 Ci/mmol) was from New

England Nuclear. Ion exchange resin AG50WX-8, lipopolysaccharide (LPS) from *Escherichia coli*, N^G -methyl-L-arginine (L-NMA), N^{ω} -nitro-L-arginine (L-NNA) and N^{ω} -nitro-L-arginine methyl ester (L-NAME) and all other reagents were from Sigma.

2.2. Animals

Samples of *A. pallida* were collected from Walsingham Pond, Bermuda. The corals *Madracis mirabilis*, *Madracis decactis*, and *Montastrea franksi* were collected from sites in Bermuda's North Lagoon; the coral *Agaricia sp.* was collected from Harrington Sound. Anemones were maintained at 25°C in 100 ml glass petri dishes in low nutrient seawater with a 12 h light/dark photoperiod and were acclimatised for at least 48 h prior to use. Individuals of 5 mm oral disc diameter or above were used for experiments. Coral tissue was extracted within 4 h of collection. Pre-treatment with the immune stimulant LPS was achieved either by injection of 20 μg directly into the anemones oral cavity or immersion of whole anemones in a solution of 2 $\mu\text{g}/\text{ml}$ in sea water 24 h prior to each experiment.

2.3. Determination of NOS activity

Individual anemones were rinsed with 2 ml ice-cold homogenization buffer (HB), 50 mM HEPES, 1 mM EDTA, pH 7.4, and homogenized in an equal volume of buffer using a 15 ml glass Potter homogenizer. The homogenate was centrifuged for 10 min at 16 000 RCF and the resulting supernatant collected. Determination of NOS activity was achieved by monitoring conversion of [^3H]arginine to [^3H]citrulline using a modification of the method of Bredt and Snyder (1989). For routine assays, 100 μl of cytosol was diluted with 100 μl HB. The incubation was initiated by the addition of 100 μl pre-mixed cofactor. Final concentrations of cofactors in the incubation were: 1 mM NADPH, 1.25 mM CaCl_2 , 1 mM dithiothreitol, 10 μM tetrahydrobiopterin, 10 units/ml of calmodulin and 20 μM L-arginine (including 12.5 pM [^3H]arginine (contained in a volume of 0.5 μl of 1 mCi/ml stock)). Following incubation at 25°C for 30 min, reactions were terminated by addition of 900 μl ice-cold stop solution (100 mM HEPES, 10 mM EDTA, pH 5.5). Entire incu-

bation volumes were applied to a 1 ml column of Dowex AG50WX-8 (Na^+ form) and [^3H]citrulline eluted with 3 ml ice-cold H_2O with pulse centrifugation at 1310 $\times g$.

A 1 ml aliquot of each eluant was added to 5 ml Ultima Gold scintillant and [^3H]citrulline quantified by liquid scintillation counting. Enzyme activity is expressed as pmoles [^3H]citrulline formed per μg protein per minute incubation. Protein was quantified following the method of Bradford (1976) using bovine serum albumin as standard. The results obtained are typically expressed as means \pm S.D. of triplicate determinations.

Kinetics of the conversion of arginine to citrulline were investigated with substrate concentrations in the range 0.01–1000 μM . V_{max} and K_m values were derived by means of Lineweaver–Burke analysis.

2.4. NADPH-diaphorase histochemistry

In NADPH diaphorase (NADPHd) staining, the reduction of the chromagen nitroblue tetrazolium (NBT) to produce a coloured histochemical product was used to localise NOS activity. Whilst not all NADPHd activity is due to NOS (Matsumoto et al., 1993), fixation of tissue in aldehyde increases the specificity so that the majority of NADPHd staining is co-localised with NOS. The NADPHd technique is widely accepted

as a reliable marker for the detection of multiple NOS isoforms (Weinberg et al., 1996).

Whole anemones were anaesthetised in 0.2 M MgCl_2 for 10 min to relax the body and tentacles, then rinsed thoroughly to remove residual MgCl_2 . Fixation was accomplished by immersing anemones for 1 h at 18°C in 0.5 M phosphate buffered saline, pH 7.6 containing 4% w/v paraformaldehyde and 0.5% w/v glutaraldehyde. After rinsing with PBS, anemones were cut vertically through the radial axis, then immersed in freshly prepared incubation solution containing 0.5 mM NBT, 0.1 mM dicumarol, 0.25% Triton X-100 and 1 mM NADPH in 0.5 M Tris–HCl pH 8.0. Control solutions were prepared without NADPH addition. After dark incubation at 18°C for 16 h, anemones were removed and rinsed with 0.5 M Tris–HCl before treatment with 100% glycerol then 70% ethanol to clear the tissue. A 21 h automated tissue-processing program (Shandon Hypercentre 2) was followed prior to embedding in fibrowax and sectioning by microtome following standard procedures.

In some experiments, whole anemones were pre-treated with LPS 24 h prior to NADPHd staining and the staining pattern compared to untreated anemones.

3. Results

3.1. Characterisation of enzyme activity

The formation of [^3H]citrulline was linear with time for up to 40 min using standard reaction mixtures containing 20 μM arginine. Subsequent reactions were terminated after 30 min incubation. The relationship between protein concentration and citrulline generation was linear up to 25 μg protein per assay (Fig. 1). The kinetics of this enzymatic activity are characterised by a K_m of 19.04 μM and a V_{max} of 2.96 pmol/min per μg protein (Fig. 2).

The arginine analogues L-NMA, D-NMA, L-NAME and L-NNA, and the arginase inhibitors L-valine and L-ornithine were pre-incubated with anemone cytosol for 10 min and NOS activity measured as described. The effects of these pre-incubations are shown in Fig. 3. Formation of [^3H]citrulline decreased with increasing concentrations of L-NMA (Fig. 4). Concentrations of L-NMA of 500 and 1000 μM reduced citrulline

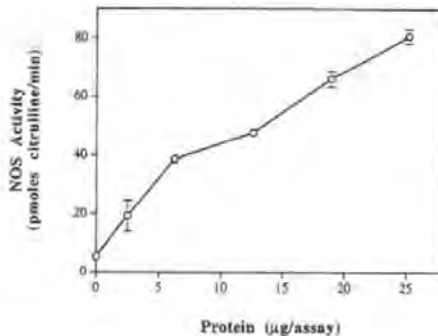


Fig. 1. The effect of protein concentration on production of [^3H]citrulline. Points represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means.

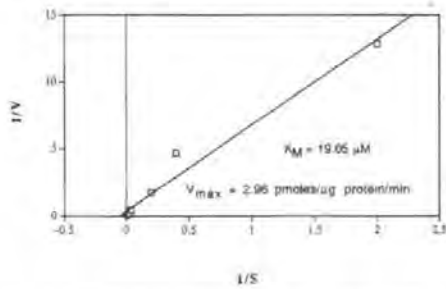


Fig. 2. Kinetics of *A. pallida* NOS activity. Production of ^3H -citrulline was measured as a function of ^3H -arginine concentration. The Lineweaver-Burke transformation of the resulting data is displayed, along with the resulting estimates of K_m and V_{\max} values.

activity by 70 and 75%, respectively. At inhibitor concentrations below 500 μM , little inhibition was evident.

Activity was completely abolished by prior heating of the homogenate to 60°C for 2 min (data not shown). The NOS activity of whole and soluble homogenate fractions gave values of 0.16 ± 0.01 pmol/min per μg protein and $0.41 \pm$

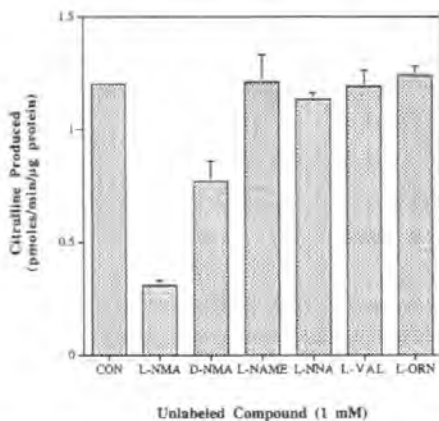


Fig. 3. Effect of arginine analogues and arginase inhibitors on the production of ^3H -citrulline. *A. pallida* homogenates were pre-incubated in 1 mM concentrations of each of the indicated compounds for 10 min prior to addition of ^3H -arginine and cofactors. Points represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means.

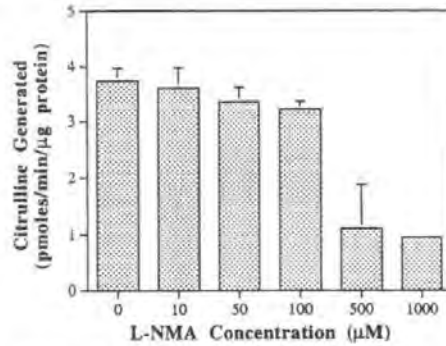


Fig. 4. Dose response relationship between the addition of L-NMA and ^3H -citrulline production. The indicated concentrations of L-NMA were added to aliquots of cytosol; assays were then performed as described in Section 2. Points represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means.

0.01 pmol/min per μg protein, respectively, indicating a predominantly cytosolic location for the enzyme activity.

In Table 1 the cofactor requirements of the enzyme preparation are shown. Addition of NADPH alone in the absence of other exogenous cofactors increased citrulline production by 17.7% relative to cytosol without exogenous addition of cofactors. Addition of CaCl_2 reduced enzyme activity by 22% relative to samples untreated with cofactors.

NOS activity was measured in anemones collected at various times from sites in Walsingham Pond, Bermuda, as well as from a variety of coral

Table 1
Effect of the addition of cofactors to anemone cytosol^a

Condition	^3H -citrulline (pmol/min per μg protein)
Full cofactor addition	0.55 ± 0.01
No cofactor addition	0.41 ± 0.01
NADPH (0.3 μM)	0.48 ± 0.01
CaCl_2 (0.375 μM)	0.32 ± 0.05
Calmodulin (150 units)	0.43 ± 0.02
DTT (0.3 μM)	0.44 ± 0.07
Biotin (0.003 μM)	0.40 ± 0.02

^a Individual chemicals were added in concentrations equivalent to those in the standard cofactor mix.

Table 2
Basal NOS activity in pmol [^3H]citrulline/ μg per min in a variety of coral species and in *A. pallida* collected from Walsingham Pond, Bermuda

Species	[^3H]citrulline production (pmol/min per μg protein)
<i>Madracis mirabilis</i>	0.24 ± 0.06
<i>Madracis decactis</i>	0.18 ± 0.01
<i>Agaricia</i> sp.	0.44 ± 0.08
<i>Montastrea franksi</i>	1.32 ± 0.23
<i>Aiptasia pallida</i>	0.80 ± 0.26

species (Table 2). The mean activity in anemones and corals was similar, typically in the range 0.50–1.16 pmol/min per μg protein.

The presence of inducible NOS activity was investigated by pre-treatment of anemones with the immune stimulant LPS, a treatment previously shown to increase iNOS activity in invertebrate species (Radomski et al., 1991). Pre-

treatment with LPS, either by injection of 20 μg directly into the oral cavity or by immersion for up to 24 h in a solution of 2 $\mu\text{g}/\text{ml}$ caused no discernible alteration in NOS activity of whole homogenate.

3.2. Histochemical localisation of NADPH diaphorase activity

Histochemical localisation of NADPHd activity revealed staining in the epidermis and at in the cells occurring at the edges of the mesoglea (Fig. 5). Tentacle sections show a similar staining pattern. Animals treated with NADPHd control incubation medium were entirely unstained. The epithelium in *A. pallida* is composed of three strata: an outer wide stratum comprising the main portions of the supporting cells interspersed with gland and sensory cells, a nervous stratum adjacent to the bases of these cells, and an innermost muscle stratum next to, or embedded in, the

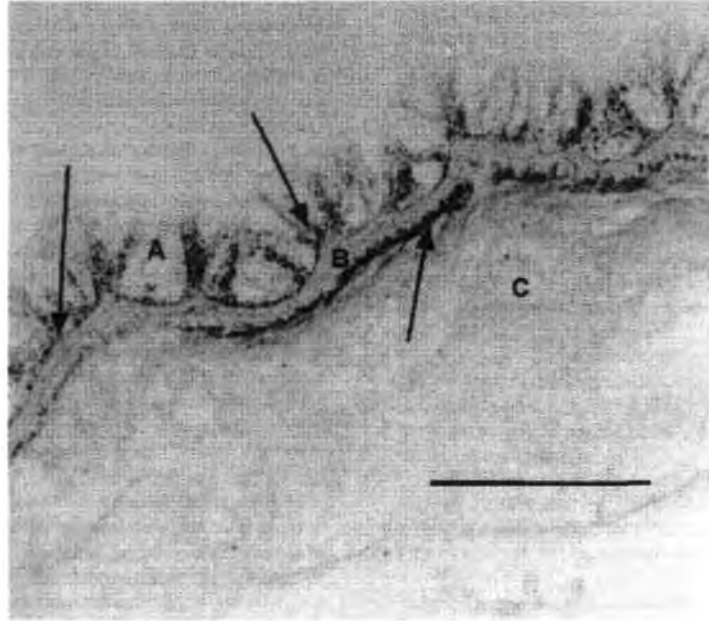


Fig. 5. NADPHd staining of a cross section of body wall of *A. pallida*. Arrows indicate staining in epidermis, and adjacent to the upper and lower surfaces of the mesoglea. (A) Epidermis; (B) mesoglea; (C) gastrodermis. Arrows indicate staining. Scale bar = 0.25 mm.

mesoglea (Hyman, 1940). The pattern shown in Fig. 5 is consistent with patchy staining of the epidermis. Bands of staining are visible at the outer and inner surfaces of the mesoglea, possibly representing nervous and muscle stratum respectively. A few zooxanthellae appeared to have taken up stain in some samples (examples not shown). Pre-incubation of whole anemones with LPS did not alter the pattern of staining. In particular, staining was absent from the mesenteric filaments, which usually contain high numbers of immunocytes (Smith and Hutton, 1995).

4. Discussion

The results presented here indicate that NOS activity is present in the body tissues of the tropical anemone *A. pallida* and in a variety of coral species. The activity of the enzyme when measured by the formation of [3 H]citrulline from [3 H]arginine indicated a basal level in whole body homogenate of 0.50–1.16 pmol pmol/min per μ g protein. This is similar to levels previously reported in rat heart homogenate using similar methods of detection (Giraldez and Zweier, 1998). The possibility that formation of [3 H]citrulline was occurring through the activity of arginase and ornithine transcarbamylase, which can indirectly synthesise L-citrulline from L-arginine (Blachier et al., 1991), was ruled out by the lack of inhibition seen with the arginase inhibitors L-valine and L-ornithine. This is an important consideration when working with a whole body homogenate in which multiple inter-related enzymatic pathways may be present. The K_m of 19.05 μ M calculated for the enzyme was higher than previously reported values of 2.9 μ M for mammalian constitutive (Pollock et al., 1991) and 2.5 μ M for invertebrate inducible (Colasanti et al., 1995) forms of the enzyme. In addition, the V_{max} value obtained was lower than other reports at 2.96 pmol/min per μ g protein. It could be that some aspect of the homogenization procedure consistently led to a high substrate requirement of the enzyme in this preparation.

The fact that enzyme activity was not affected by pre-treatment with LPS, taken together with the cytosolic location, suggest that the enzyme activity bears some similarity to mammalian

nNOS. The rank order of inhibition seen for competitive arginine analogues is in agreement with this, although the relative potency of L-NMA and L-NNA as inhibitors, especially of constitutive nNOS are reportedly highly dependent on experimental conditions (Klatt et al., 1994). Formation of [3 H]citrulline was inhibited by L-NMA in a dose-dependent manner, with residual activity remaining even in the presence of high concentrations of L-NMA. This lack of complete inhibition even when L-NMA is present at concentrations higher than the competing substrate arginine, has been noted by others (Griffiths and Gross, 1996).

Comparison of enzyme activity with and without cofactor addition indicate that 75% activity can be achieved without exogenous cofactor addition (assuming maximum activity is achieved when full cofactor mix is added). These findings suggest endogenous cofactors are present in the cytosolic fraction in concentrations sufficient to support close to maximal activity. These findings are similar to those of Elphick et al. (1993) who found NOS activity in locust brain extracts to be reduced from 80% of the maximum when NADPH was omitted. This indicates that there is sufficient endogenous NADPH to sustain the majority of the maximum activity. The reduction in enzyme activity seen when CaCl_2 was added is a striking finding, that has been noted by other researchers; Radomski et al. (1991) showed an approximately 30% reduction in activity on addition of 200 μ M Ca^{2+} . This was interpreted as evidence of the presence of a calcium independent NOS enzyme. Alternatively, such observations could be seen as being consistent with an enzyme possessing a biphasic response to this divalent cation. Experiments designed to determine which of these interpretations is correct need to be conducted. In addition, the apparent lack of calmodulin dependency shown in this work warrants further investigation using specific calmodulin inhibitors.

The functional similarities between the invertebrate enzyme reported here and mammalian NOS enzymes is worth noting, separated as they are by 500 million years of evolution. However, a direct comparison to mammalian constitutive and inducible isoforms may be inappropriate. Recent phylogenetic evidence (Hughes, 1998) indicates that the gene duplication preceding the evolution

of an immunological role for iNOS in mammalian species may have occurred after the divergence of vertebrates and invertebrates. Diversity of function and regulation would then have arisen convergently in these groups.

It has been suggested that NO has evolved to be such a ubiquitous and successful signaling molecule because of the regulatory opportunities offered by the unique active site structure of NOS, located at the interface of the dimeric structure (Crane et al., 1997). This location offers the opportunity for substrate and effector molecules such as bipterin and calmodulin to modulate catalysis by affecting associations between domains and subunits and is likely to be strongly conserved.

Although the results presented here give no direct information on the functional role of NOS in *A. pallida*, it is possible to speculate on the likely role of the enzyme based on studies of other invertebrate species. The distribution of NOS activity throughout the outer epithelial layer and in cells adjacent to the inner and outer edges of the mesoglea shown by NADPHd staining is compatible with a broad involvement of NOS in both sensory and motor activities. Elofsson et al. (1993), using essentially similar techniques to those used here, found only non-selective staining in *Aiptasia* sp. and in the similar cnidarian *Hydra oligactis* and suggested that the distribution of NADPHd positive cells might be restricted to invertebrates having well developed nervous systems. However, in many invertebrate species in which NOS has been studied, the enzyme appears in the central and peripheral nervous system. In molluscs, there has been an apparent evolution in the involvement of NO in muscle cell physiology (Martinez, 1995). The more primitive groups such as the polyplacophora show NADPHd staining only in buccal muscle cells. In other phyla, positivity is apparent in the neurons innervating these muscles, which may indicate a switch in the site of NO-mediated control of muscle motility. NOS activity has been shown to be a controlling factor in the primitive olfactory feeding response of the cnidarian *Hydra vulgaris*, considered the most primitive multicellular organism to possess a nervous system (Colasanti et al., 1997). The response consists of tentacular writhing and mouth opening and is similar to the feeding response seen in *A. pallida*.

Involvement of NO in the regulation of smooth muscle tone has been demonstrated in the echinoderm *Asterias rubens* (Elphick and Melarange, 1998) in which relaxation of the cardiac stomach was induced using the NO-donor chemicals *S*-nitrosoglutathione (SNOG) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). Induction of NOS by pre-treatment with LPS occurs in molluscan haemocytes (Smith et al., in press; Conte and Ottaviani, 1995) and a central role for NO proposed in the integration of inflammation and stress responses in these cell types (Galloway and Depledge, 2000). The exact functional role of the NO-cGMP signaling pathway in the physiological responses of *A. pallida* merits further study.

In conclusion, we believe this to be the first characterisation of a constitutive NOS activity in the tropical marine anemone *A. pallida*. NOS activity was also observed in a variety of coral species. The cytosolic enzyme is located predominantly in the outer cell layers of the tentacles and body wall. A preliminary characterisation of the enzyme's properties show it to share some functional characteristics with mammalian nNOS. Further comparison between the properties and functional roles of mammalian and invertebrate NOS isoforms may give clues to the evolutionary origins of this enzyme.

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References

- Bascal, Z.A., Montgomery, A., Holden-Dye, L., Williams, R.G., Walker, R.J., 1995. Histochemical mapping of NADPH diaphorase in the nervous system of the parasitic nematode *Ascaris suum*. *Parasitology* 110, 625–637.
- Blachier, F., Darcy-Vrillon, B., Sener, A., Duce, P., Malaisse, W.J., 1991. Arginine metabolism in rat erythrocytes. *Biochim. Biophys. Acta* 1092, 304–310.

- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Bredt, D.S., Snyder, S.H., 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* 86, 9030–9033.
- Leake, L.D., Moroz, L.L., 1996. Putative nitric oxide synthase (NOS) containing cells in the central nervous system of the leech *Hirudo medicinalis*. *Brain Res.* 723, 115–124.
- Chen, Y., Rosazza, J.P., 1994. A bacterial nitric oxide synthase from a *Nocardia* species. *Biochem. Biophys. Res. Commun.* 203, 1251–1258.
- Colasanti, M., Lauro, G.M., Venturini, G., 1995. NO in hydra feeding response. *Nature* 374, 505.
- Colasanti, M., Venturini, G., Merante, A., Musci, G., Lauro, G., 1997. Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *J. Neurosci.* 17, 493–499.
- Conte, A., Ottaviani, E., 1995. Nitric oxide synthase activity in molluscan hemocytes. *FEBS Lett.* 365, 120–124.
- Crane, B.R., Arvai, A.S., Gachhui, R., Wu, C., Ghosh, D.K., Getzoff, E.D., Stuehr, D.J., Tainer, J.A., 1997. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* 278, 425–431.
- Delledonne, M., Xia, Y., Dixon, R.A., Lamb, C., 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature* 394, 585–588.
- Elofsson, R., Carlberg, M., Moroz, L., Nezhlin, L., Sakharov, D., 1993. Is nitric oxide produced in invertebrate neurons? *Neuroreport* 4, 279–282.
- Elphick, M.R., Green, I.C., O'Shea, M., 1993. Nitric oxide synthesis and action in the invertebrate brain. *Brain Res.* 619, 344–346.
- Elphick, M.R., Melarange, R., 1998. Nitric oxide function in an echinoderm. *Biol. Bull.* 194, 260–266.
- Galloway, T.S., Depledge, M.H., 2000. Effects of immunotoxic chemicals in invertebrates. *Mar. Poll. Bull.* (in press).
- Garthwaite, J., 1991. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.* 14, 60–67.
- Giraldez, R.R., Zweier, J.L., 1998. An improved assay for measurement of nitric oxide synthase activity in biological tissues. *Anal. Biochem.* 261, 29–35.
- Griffiths, O.W., Stuehr, D.J., 1995. Nitric oxide synthases. Properties and catalytic mechanisms. *Annu. Rev. Physiol.* 57, 707–736.
- Griffiths, O.W., Gross, S.S., 1996. Inhibitors of nitric oxide synthases. In: Feelisch, M., Stamler, J.S. (Eds.), *Methods in Nitric Oxide Research*. Wiley, Chichester, UK, pp. 187–208.
- Hughes, A.L., 1998. Protein phylogenies provide evidence of a radical discontinuity between arthropod and vertebrate immune systems. *Immunogenetics* 47, 283–296.
- Hyman, L.H., 1940. Metazoa of the tissue grade of construction—the radiate phyla—phylum Cnidaria, Vol. 1. In: Boell, E.J. (Ed.), *The Invertebrates Protozoa through to Ctenophora*. McGraw-Hill, New York, pp. 365–661.
- Janssens, S.P., Shimouchi, A., Quertermous, T., Bloch, D.B., Bloch, K.D., 1992. Cloning and expression of a cDNA-encoding human endothelial-derived relaxing factor nitric oxide synthase. *J. Biol. Chem.* 267, 14519–14522.
- Klatt, P., Schmidt, K., Brunner, F., Mayer, B., 1994. Inhibitors of brain nitric oxide synthase: binding kinetics, metabolism and enzyme inactivation. *J. Biol. Chem.* 269, 1674–1680.
- Martinez, A., 1995. Nitric oxide synthase in invertebrates. *Histochem. J.* 27, 770–776.
- Matsumoto, T., Nakane, M., Pollock, J.S., 1993. A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. *Neurosci. Lett.* 155, 61–64.
- Moncada, S., Higgs, A., 1993. The L-arginine-nitric oxide pathway. *N. Eng. J. Med.* 329, 2002–2012.
- Nathan, C., Xie, Q., 1994. Nitric oxide synthases: roles, tolls and controls. *Cell* 78, 915–918.
- Pollock, J.S., Forstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H., Nakane, M., Murad, F., 1991. Purification and characterisation of particulate endothelium derived relaxation factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* 88, 10480–10484.
- Radomski, W., Martin, J.F., Moncada, S., 1991. Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (*Limulus polyphemus*). *Phil. Trans. R. Soc. Lond. B* 334, 129–133.
- Salleo, A., Musci, G., Barra, P.F., Calabrese, L., 1996. The discharge of acontial nematocytes involves the release of nitric oxide. *J. Exp. Biol.* 199, 1261–1267.
- Smith, V.J., Hutton, D.M., 1995. Measurement of extracellular reactive oxygen species in Anthozoans and other marine invertebrates by cytochrome C reduction. *Techniques in Fish Immunology* 4, 27–35.
- Smith, K.L., Galloway, T.S., Depledge, M.H., 1999. Neuroendocrine biomarkers of pollution-induced stress in the marine bivalve *Mytilus edulis*. *Sci. Total. Environ.* (in press).
- Stuehr, D.J., 1997. Structure function aspects of the nitric oxide synthases. *Annu. Rev. Pharmacol. Toxicol.* 18, 707–729.

- Stuehr, D.J., Griffiths, O.W., 1992. Mammalian nitric oxide synthases. *Adv. Enzymol.* 65, 287–347.
- Verrill, V.E., 1900. Additions to the anthozoa and hydrozoa of the Bermudas. *Trans. Conn. Acad. Arts. Sci.* 10, 551–572.
- Weinberg, R.J., Valschanoff, J.G., Schmidt, H.H., 1996. The NADPH diaphorase histochemical stain. In: Feelisch, M., Stamler, J.S. (Eds.), *Methods in Nitric Oxide Research*. Wiley, New York, pp. 237–248.

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Nitric Oxide and Cnidarian-Dinoflagellate Symbioses: Pieces of a Puzzle¹HENRY G. TRAPIDO-ROSENTHAL,² KATHERINE H. SHARP,³ TAMARA S. GALLOWAY,⁴ AND CLARE E. MORRALL⁵

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SYNOPSIS. The presence of nitric oxide synthase (NOS) activity is demonstrated in the tropical marine cnidarian *Aiptasia pallida* and in its symbiotic dinoflagellate algae, *Symbiodinium bermudense*. Enzyme activity was assayed by measuring the conversion of arginine to citrulline. Biochemical characterization of NOS from *Aiptasia* was characterized with respect to cellular localization, substrate and co-factor requirements, inhibitors, and kinetics. In response to acute temperature shock, anemones retracted their tentacles. Animals subjected to such stress had lower NOS activities than did controls. Treatment with NOS inhibitors caused tentacular retraction, while treatment with the NOS substrate L-arginine inhibited this response to stress, as did treatment with NO donors. These results provide a preliminary biochemical characterization of, and suggest a functional significance for, NOS activity in anthozoan-algal symbiotic assemblages.

INTRODUCTION

Nitric oxide (NO)

Over the past several years, NO has been recognized as a molecule of fundamental significance in mediating a variety of interactions between cells. It plays important roles as a weapon in the cellular defenses of metazoans (Nathan and Hibbs, 1991), as a modulatory molecule in the chemosensory modality of olfaction (Breer and Shepherd, 1993), as a mediator of a variety of secretory processes, including those for the export of molecules such as amino acids and peptides, and ions such as bicarbonate (Schmidt and Walter, 1994), and, most famously if not most importantly, as a regulator of vascular processes (Burnett *et al.*, 1992). Although the role of NO in these phenomena has been best characterized in

vertebrates, and usually in mammals, NO is implicated in the chemosensory responsiveness of aquatic invertebrates from taxa as diverse as cnidarians (Colasanti *et al.*, 1995), molluscs (Elphick *et al.*, 1995a) and arthropods (Elphick *et al.*, 1995b). Furthermore, each of the listed functions can be seen as being of potential fundamental importance in the establishment, maintenance, and termination of endosymbiotic relationships such as those that exist between anthozoans and photosynthetic dinoflagellates.

NO differs from many molecules used in cellular signaling in several fundamental aspects. Because it passes through cellular membranes, it cannot be stored in vesicles and exocytosed upon demand. Rather than being made in advance, NO must be generated on an as-needed basis. Furthermore, due to the transparency of biological membranes to this molecule, its many effects are not mediated by non-covalent, spatially complementary interactions with cell surface receptors. Instead, NO exerts its effects by covalently interacting with molecular oxygen, the superoxide anion, transition metals, and thiol groups (Stamler, 1994). Proteins that complex with metal ions or that contain thiol groups are thus candidates for the sites at which NO can exert a physiologically meaningful function.

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Nitric oxide synthase (NOS)

The fact that NO must be produced on an as-needed basis has led to extensive studies of NOS, the enzyme(s) responsible for the generation of this molecule. Although it is now known that there are multiple isoforms of NOS, they are all currently grouped under the single Enzyme Commission number 1.14.13.39. The first NOS to be isolated (Bredt and Snyder, 1990) and cloned, sequenced, and functionally expressed (Bredt *et al.*, 1991) came from a mammalian neural source (rat cerebellum). To perform its function of converting L-arginine to citrulline and NO, this NOS required as cofactors calmodulin, NADPH, and flavin mononucleotides. Analysis of cDNA and deduced amino acid sequences demonstrated that this enzyme had a high degree of resemblance to cytochrome P-450 reductase, an enzyme involved in electron transfer reactions that also requires flavins and NADPH as cofactors.

Subsequently, NOSs from other tissue types and other organisms have been functionally and molecularly characterized. As enough data have accumulated from mammalian sources, comparisons have shown that the functional, antigenic, and sequential similarities between the various NOSs are more striking than the differences (Nathan and Xie, 1994). Enough differences exist, however, to group the NOSs into two categories. One category, which includes the neuronal NOS first characterized by Bredt and colleagues, is constitutively expressed, and is calmodulin dependent. The other category is inducible (or, more accurately, perhaps, subject to dramatic up-regulation) and is not as dependent on calmodulin (Maretta, 1994). Sequential homologies are stronger within constitutive and inducible categories than between them, but the isoforms are still clearly structurally similar. The striking functional and structural conservatism of the NOSs that have been characterized to date make it plausible to hypothesize that many of the NOSs from non-mammalian sources may be similar to them. Studies of NOSs from lower vertebrates (Liepe *et al.*, 1994), molluscs (Elphick *et al.*, 1995a), and arthropods (Elphick *et al.*,

1995b) so far seem to corroborate this hypothesis.

Chemical mediation of cnidarian-algal symbioses

From the earliest recognition of the existence of cnidarian-algal symbioses, it was hypothesized that the partners in the relationship exchanged chemicals (reviewed by Shick, 1991). Throughout this century, there have been numerous demonstrations of such metabolic interchanges, which have been summarized in a number of recent reviews (Trench, 1971a, b, c; Muscatine and Porter, 1977; Cook, 1983; Shick, 1991). Much of this work has focussed on the transfer of photosynthetically-generated carbohydrate from algal symbiont to cnidarian host. Movement of nitrogen from host to symbiont, usually in the form of ammonium, and the transfer of amino acids between the partners have also been studied. Of particular interest are studies by McCauley (1986, 1987, 1988, 1991) demonstrating that *Chlorella*, the genus of algae that live in symbiosis with *Hydra viridis-sima*, can internalize exogenous amino acids, and that amino acid transport rates are higher in algae that have been freshly isolated from a symbiotic host than they are in algae that have been maintained in culture. In a related line of investigation, Ferrier (1992) showed that the free amino acid pool of symbiotic dinoflagellate algae (*Symbiodinium*) freshly isolated from the sea anemone host *Aiptasia pallida* was dominated by the basic amino acid arginine, while this amino acid was present in only very low concentrations in conspecific algae that had been maintained in culture.

Compared to studies of nutritional fluxes, the study of chemical interactions between host and symbiont that are fundamentally informational in nature has a shorter and more recent history. Fitt (1984, 1985) has demonstrated that amino acids, presumably host-derived, can serve as chemoattractants for free-living cells of *Symbiodinium microadriaticum* (as all strains of this dinoflagellate were then called); his results can be interpreted as a demonstration of host-to-symbiont chemical communication. A number of investigators have sought to identify

and characterize a host-derived chemical or mixture of chemicals, known as host factor (HF), that can induce symbionts to export photosynthetically-generated carbohydrate. Their studies have produced a body of contradictory results, with HF being described as being both heat stable and heat labile (Muscantine, 1967; Muscantine *et al.*, 1972; Sutton and Hoegh-Guldberg, 1990), as being both a large and a small molecule (Cook and Orlandini, 1992), as being both present and absent in dinoflagellate-free anemones (Cook and Orlandini, 1992), and as both enhancing and not enhancing carbon fixation (Trench, 1971c; Masuda *et al.*, 1994). Gates *et al.* (1995) demonstrated that a crude aqueous extract of tissue from the coral *Pocillopora damicornis* both enhances the fixation of carbon by, and induces the export of fixed carbon from, *Symbiodinium* isolated either from *P. damicornis* or from the sea anemone *Aiptasia pulchella*. They characterized the amino acid composition of this crude extract, and demonstrated that a synthetic free amino acid mixture based upon this composition was capable of mimicking the carbon fixation and exporting effects of the crude extract. In similar experiments, Bester *et al.* (1997) demonstrated that an amino acid mixture qualitatively and quantitatively representative of the free amino acid composition of *A. pallida* is capable of converting the low-capacity, high-affinity amino acid transport of free-living, cultured *Symbiodinium bermudense* cells to the high-capacity, low-affinity transport characteristic of cells of *S. bermudense* that have been freshly isolated from a symbiotic relationship. Experiments such as these have clearly demonstrated that identified chemical signals from the host can induce changes in the cellular physiology of photosynthetic symbionts. The ways in which these signals are transduced by the algae remain to be elucidated. Also unknown is whether chemicals that function as signals, rather than as nutrients, pass from the symbionts to the host organism.

Research goal

The goal of our research is to advance our understanding of the chemical communication between the members of cni-

darian-dinoflagellate symbioses. In the following sections, we describe work done to date to test the hypothesis that NO may be an important signal molecule in these assemblages.

MATERIALS AND METHODS

Chemicals

Radiolabeled L-arginine (^3H , 40Ci/mmol) was obtained from New England Nuclear, and the liquid scintillation cocktail UltimaGold was from Packard Instrument Corporation. All other reagents were from Sigma Chemical Company.

Organisms

Samples of *A. pallida* were collected from Walsingham Pond, Bermuda. Anemones were maintained at 25°C in 100 ml glass petri dishes in low nutrient seawater (collected from a depth of 10 m in the Sargasso Sea, 175 km southeast of Bermuda) with a 12 hr light/dark photoperiod; they were acclimatized for at least 48 hr before use. Individuals of 5 mm oral disc diameter or above were used for experiments. Symbiotic specimens of *Symbiodinium bermudense* were isolated from *A. pallida* by mechanical homogenization of the symbiotic assemblage, with subsequent removal of the algae from the homogenate by repeated centrifugation and filtration. Algae were ultimately suspended in sterile F/2 medium (Guillard, 1975). Cultured specimens of *S. bermudense* were obtained from the Provasoli-Guillard National Collection of Marine Phytoplankton, and maintained at a concentration of 250,000 cell/ml in sterile F/2 medium at 26°C with a 12 hr photoperiod. These cultures were originated from algae isolated from specimens of *A. pallida* that had been collected from Walsingham Pond, Bermuda.

Determination of NOS activity

Individual anemones were rinsed with 2 ml and homogenized in an equal volume of homogenization buffer (HB: 50 mM HEPES, 1 mM EDTA, pH 7.4) using a 15-ml glass Potter homogenizer. The homogenate was centrifuged for 10 min at 16,000 RCF and the resulting supernatant collected. NOS activity was determined by monitor-

ing conversion of ^3H -arginine to ^3H -citrulline using a modification of the method of Bredt and Snyder (1989), as described by Morrall *et al.* (2000). Briefly, 100 μl of homogenate supernatant was brought to 200 μl with HB. The incubation was initiated by adding 100 μl of HB containing cofactors and radiolabel. Final concentrations of cofactors in the incubation were: 1 mM NADPH, 1.25 mM CaCl_2 , 1 mM dithiothreitol, 10 μM tetrahydrobiopterin, 10 units/ml of calmodulin, and 20 μM ^3H -L-arginine. Following incubation at 25°C for 30 min, reactions were terminated by addition of 900 μl ice-cold stop solution (100 mM HEPES, 10 mM EDTA, pH 5.5). Entire incubation volumes were applied to a 1-ml column of Dowex AG50WX-8 (Na^+ form) and ^3H -citrulline was eluted with 3 ml ice-cold H_2O with pulse centrifugation at $1,310 \times g$. A 1-ml aliquot of eluant was mixed with 5 ml Ultima Gold, and ^3H -citrulline quantified by liquid scintillation spectrophotometry. Enzyme activity is expressed as pmoles ^3H -citrulline formed per μg protein per minute incubation, with protein being quantified by means of the dye-binding assay of Bradford (1976), using bovine serum albumin as standard. The results obtained are expressed as means \pm SD of determinations on multiple anemones; typically, each data point represents NOS activity from three specimens. Kinetics of the conversion of arginine to citrulline were investigated with substrate concentrations in the range 0.01–1,000 μM . V_{max} and K_m values were derived by Lineweaver-Burke analysis. Potential inhibitors of NOS activity were included in incubation media at concentrations indicated in legends to figures.

To determine NOS activity in *Symbiodinium*, algae, either isolated from anemones or taken from culture, were first rinsed with ice-cold HB. They were then sonicated in 1 ml of HB, in an ice-jacketed 1.5-ml microcentrifuge tube with a Heat Systems sonicator. The sonicate was centrifuged for 10 min at 16,000 RCF and the resulting supernatant collected. NOS activity was then determined as described above.

Effects of temperature stress on sea anemone behavior and NOS activity

The short-term effects of the acute thermal stressing of animals on NOS activity was investigated by incubating anemones at either 17°C for 120 min, or 33°C for either 30 or 120 min, prior to homogenization and assay; results were compared with those obtained from control animals maintained at 25°C . Long-term effects were investigated by incubating anemones in water that was slowly warmed over a period of several days, prior to homogenization and assay. In both experiments, anemone behavior was observed during these incubations.

Effects of NOS substrate, NOS inhibitors, and NO donors on sea anemone behavior

Effects of NOS inhibitors and NO donors on anemone behavior were examined by observing anemones that were incubated in seawater containing 1 mM concentrations of either the NOS substrate L-arginine, the NOS inhibitor N^G -monomethyl-L-arginine (NMMA), or the NO donor S-nitrosoglutathione (SNOG), at both 25°C and 28°C . Control incubations contained no additions.

RESULTS

Preliminary observations: NOS activity in anemones

In preliminary time-course experiments, the generation of ^3H -citrulline was linear with time for up to 40 min using standard reaction mixtures containing 20 μM arginine (data not shown). In subsequent experiments, incubations were standardized at 30 min. The relationship between protein concentration and citrulline generation was linear up to 25 μg protein per assay (Fig. 1; Morrall *et al.*, 2000). Activity was completely abolished by prior heating of the homogenate to 60°C for 2 min (data not shown). The NOS activity of whole and cytosolic homogenate fractions were essentially equal, indicating a predominantly cytosolic location for the enzyme activity (Fig. 2). The kinetics of this enzymatic activity are characterized by a K_m of 19.04 μM and a V_{max} of 2.96 pmol/min/ μg protein (Fig. 3; Morrall *et al.*, 2000).

The arginine analogues N^G -monomethyl-

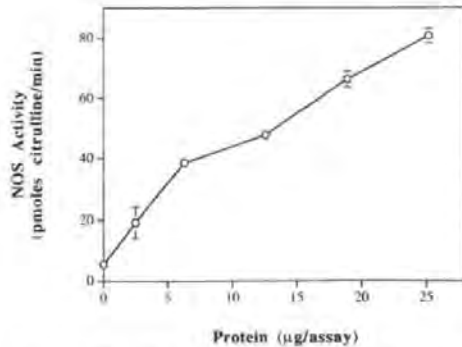


FIG. 1. The effect of protein concentration on production of ^3H -citrulline. Points represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

L-arginine acetate (L-NMMA), D-NMMA, N^G -nitro-L-arginine methyl ester (L-NAME), and N^G -nitro-L-arginine (L-NNA), and the arginase inhibitors L-valine and L-ornithine were pre-incubated with anemone cytosol for 10 min at up to 1 mM concentrations, after which NOS activity was determined. The effects of treatment with these potential inhibitors are shown in Figure 4 (Morrall *et al.*, 2000). Formation of ^3H -citrulline decreased with increasing con-

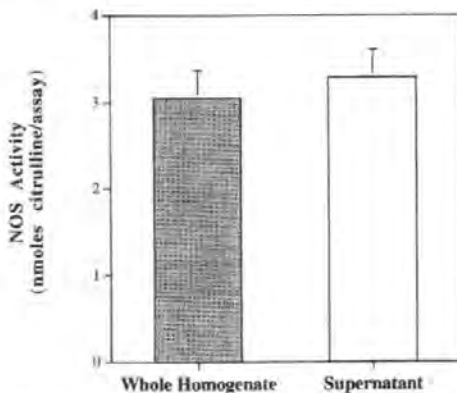


FIG. 2. Localization of NOS activity. Homogenates of *A. pallida* were split into two fractions, one of which was assayed directly (Whole Homogenate), while the other was centrifuged to remove membranous material. Bars represent the means and standard errors of triplicate determinations.

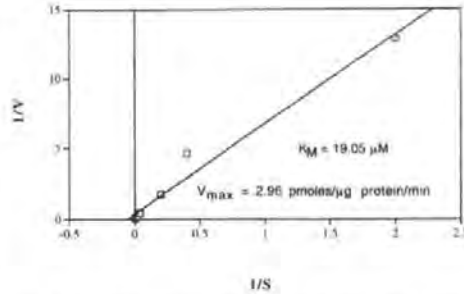


FIG. 3. Kinetic parameters of *A. pallida* NOS activity. Production of ^3H -citrulline was measured as a function of ^3H -arginine concentration. The Lineweaver-Burke transformation of the resulting data is displayed, along with the resulting estimates of K_M and V_{max} values (from Morrall *et al.*, 2000, with permission from Elsevier Science).

centrations of L-NMMA (Fig. 5; Morrall *et al.*, 2000). Concentrations of L-NMMA of 500 and 1,000 μM reduced citrulline activity by 70% and 75% respectively, while at inhibitor concentrations below 500 μM , little inhibition was evident.

NOS activity levels were optimized by the addition of exogenous NADPH, were unaffected by addition of exogenous tetra-

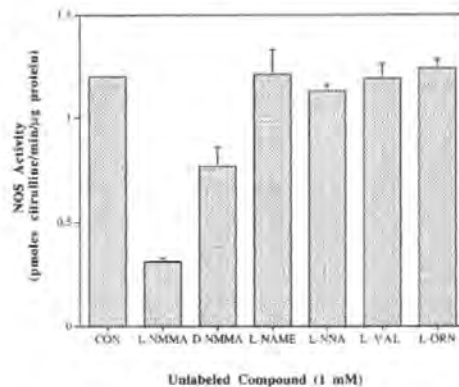


FIG. 4. Effect of arginine analogues and arginase inhibitors on the production of ^3H -citrulline. *A. pallida* homogenates were pre-incubated in 1 mM concentrations of each of the indicated compounds for 10 min prior to addition of ^3H -arginine and cofactors. Bars represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

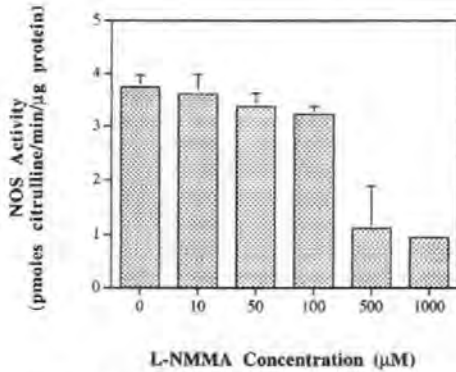


FIG. 5. Effect of increasing concentrations of L-NMMA on ^3H -citrulline production. The indicated concentrations of L-NMMA were added to aliquots of cytosol 10 min prior to assays, which were then performed as described in Materials and Methods. Bars represent the means and standard errors of triplicate determinations; where no error bars appear, standard errors were less than 5% of the indicated means (from Morrall *et al.*, 2000, with permission from Elsevier Science).

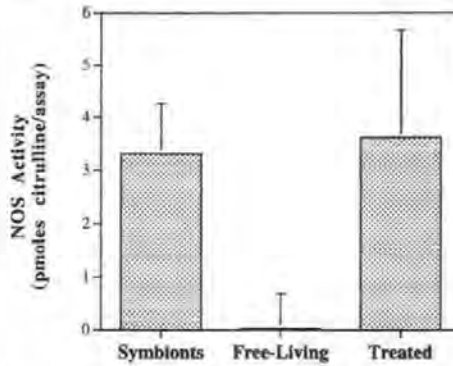


FIG. 6. NOS activity of sonicates of *S. bermudense*. Each bar represents the means and standard errors of triplicate determinations of NOS activity in sonicates of 10^8 algal cells. The bar labeled 'symbionts' represents activity in cells that were freshly isolated from *A. pallida*. The bar labeled 'free-living' represents activity in cells that had been maintained in a symbiosis-free culture in F/2 medium (Guillard, 1975). The bar labeled 'treated' represents activity in free-living cultured cells that had been pre-incubated in a mixture of free amino acids that represents the free amino acid composition of *A. pallida*.

hydrobiopterin, dithiothreitol, or calmodulin, and were reduced by the addition of exogenous CaCl_2 (Morrall *et al.*, 2000).

NOS activity in zooxanthellae

Algal cells that had been freshly isolated from *A. pallida* possessed NOS activity, whereas cells that had been maintained in non-symbiotic conditions (in culture) had little or no ability to convert arginine to citrulline (Fig. 6). However, if cultured zooxanthellae were preincubated for 48 hr in an amino acid mixture that mimicked the free amino acid concentration of *A. pallida* (Bester *et al.*, 1997), when assayed they had NOS activity comparable to cells that had just been removed from a symbiotic relationship (Fig. 6).

Effect of temperature stress on NOS activity

Transferring anemones directly from 25°C water to 33°C resulted in a pronounced retraction of the animals' tentacles. Upon homogenization and assay, either 30 min or 120 min after temperature change, NOS activities were significantly lower in temperature-stressed animals than they were in unstressed controls (Fig. 7). The

same phenomena of tentacular retraction and lowered NOS activities were seen when animals were subjected to cooler, 17°C water (Morrall *et al.*, 1998).

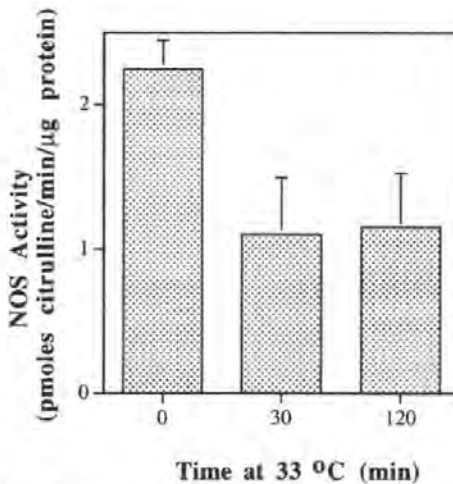


FIG. 7. Effect of an 8°C increase in temperature, for the indicated periods of time, on NOS activity in *A. pallida*. Bars represent the means and standard errors of triplicate determinations.

TABLE 1. The effect of L-NMMA, L-arginine and SNOG on Aiptasia behavior.*

Condition	25°C	28°C
Control	Fully expanded tentacles.	Tentacles and column contracted. Stubby. Small oral disc.
L-NMMA	Tentacles retracted and more flaccid than control.	Some tentacles fully retracted into column. Oral disc very small.
L-arginine	Tentacles fully expanded. Wide oral disc—not visibly different from control.	Tentacles and column fully expanded. Oral disc widely expanded.
SNOG	Tentacles fully expanded. Wide oral disc—not visibly different from control.	Tentacles and column fully expanded. Oral disc widely expanded.

* Incubations were performed and observations were made as described in *Materials and Methods*. The indicated compounds were present at 1 mM concentrations.

Effects of NOS substrate, NOS inhibitors, and NO donors on anemone behavior

The effects of incubation in the NOS substrate L-arginine, in the NOS inhibitor L-NMMA, and the NO donor SNOG are reported in Table 1. At the ambient temperature of 25°C, organisms incubated in L-NMMA were more retracted than either control anemones or anemones incubated in L-arginine or SNOG. Under conditions of elevated temperature, control anemones retracted their tentacles, whereas animals incubated with either L-arginine or SNOG appeared as fully extended as did animals at ambient temperature.

DISCUSSION

The results presented here and in the work of Morrall *et al.* (2000) indicate that NOS activity is present in the tissue of the tropical anemone *A. pallida*. The activity of the enzyme when measured by the formation of ³H-citrulline from ³H-arginine indicated a basal level in whole body homogenate of 0.50–1.16 pmol/min/μg protein (Morrall *et al.*, 2000), which is similar to levels previously reported in rat heart homogenate using similar methods of detection (Giraldez and Zweier, 1998). The possibility that formation of ³H-citrulline was occurring through the activity of arginase and ornithine transcarbamylase, which can indirectly synthesize L-citrulline from L-arginine (Blachier *et al.*, 1991) was ruled out by the lack of inhibition seen with the arginase inhibitors L-valine and L-ornithine. This is an important consideration when

working with a whole body homogenate in which multiple and related enzymatic pathways may be present. The K_m of 19.05 μM calculated for the enzyme was higher than previously reported values of 2.9 μM for mammalian constitutive (Pollock *et al.*, 1991) and 2.5 μM for invertebrate inducible (Colasanti *et al.*, 1995) forms of the enzyme. In addition, the V_{max} value obtained was lower than other reports at 2.96 pmol/min/μg protein. The differences in purification state of the enzyme preparations used in our studies and in those of other workers may account for some of these quantitative differences, and may in fact make comparisons of kinetic parameters from different studies of only marginal value.

The fact that enzyme activity was not affected by pre-treatment with bacterial lipopolysaccharide (Morrall *et al.*, 2000), taken together with the cytosolic location, suggest that the enzyme activity bears some similarity to mammalian neuronal NOS (nNOS). The rank order of inhibition seen for competitive arginine analogues is in agreement with this, although the relative potency of L-NMMA and L-NNA as inhibitors, especially of constitutive nNOS, are reportedly highly dependent on experimental conditions (Klatt *et al.*, 1994). Formation of ³H-citrulline was inhibited by L-NMMA in a dose-dependent manner, with residual activity remaining even in the presence of high concentrations of this inhibitor. This lack of complete inhibition even when L-NMMA is present at concentrations high-

er than the competing substrate arginine, has been noted by others (Griffiths and Gross, 1996).

Comparison of enzyme activity with and without cofactor addition (Morrall *et al.*, 2000) indicates that 75% activity can be achieved without exogenous cofactor addition (assuming maximum activity is achieved when full cofactor mix is added). These findings suggest that endogenous cofactors are present in the cytosolic fraction in concentrations sufficient to support close to maximal activity. These findings are similar to those of Elphick *et al.* (1993), who found that NOS activity in locust brain extracts was reduced to 80% of the maximum when NADPH was omitted. The reduction in enzyme activity seen when CaCl_2 was added is a striking finding, and has been noted by other researchers; Radomski *et al.* (1991) showed an approximately 30% reduction in activity on addition of 200 μM Ca^{2+} . This was interpreted as evidence of the presence of a calcium independent NOS enzyme. Alternatively, such observations are also consistent with an enzyme possessing a biphasic response to this divalent cation. Experiments designed to determine which of these interpretations is correct need to be conducted. In addition, the apparent lack of calmodulin dependency shown in this work warrants further investigation using specific calmodulin inhibitors.

The functional similarities between the invertebrate enzyme reported here and mammalian NOS enzymes is worth noting, separated as they are by 500 million years of evolution. However, a direct comparison to mammalian constitutive and inducible isoforms may be inappropriate. Recent phylogenetic evidence indicates that the gene duplication preceding the evolution of an immunological role for iNOS in mammalian species may have occurred after the divergence of vertebrates and invertebrates (Hughes, 1998). Diversity of function and regulation would then have arisen convergently in these groups.

Although the results presented here give no direct information on the functional role of NOS in *A. pallida*, it is possible to speculate on the likely role of the enzyme based

on studies of other invertebrate species. The demonstration by Morrall *et al.* (2000) that NOS activity, as localized by NADPH diaphorase (NADPHd) histochemistry, is distributed throughout the outer epithelial layer as well as in cells of the gastrodermal layer is compatible with a broad involvement of NOS in both sensory and motor activities. Elofsson *et al.* (1993) found only non-selective staining in *Aiptasia* sp. and in another cnidarian, *Hydra oligactis*, and suggested that the distribution of NADPHd positive cells might be restricted to invertebrates having more developed nervous systems. However, in many invertebrate species in which NOS has been studied, the enzyme appears in the central and peripheral nervous system. In molluscs, NO is apparently involved in muscle cell physiology (Martinez, 1995). In addition, individual nitergic neurons in both the buccal ganglion and cerebral ganglion of *Lymnaea stagnalis* are directly involved in that mollusc's feeding behavior (Korneev *et al.*, 1998, 1999; Park *et al.*, 1998). The polyplacophorans show NADPHd staining only in buccal muscle cells. In other phyla, activity is apparent in the neurons innervating these muscles, which may indicate a switch in the site of NO-mediated control of muscle motility. NOS activity has been shown to be a controlling factor in the olfactory feeding response of the cnidarian *Hydra vulgaris* (Colasanti *et al.*, 1997). The response consists of tentacular writhing and mouth opening and is similar to the feeding response seen in *A. pallida*.

Many of the effects of NO in invertebrates, including phenomena as diverse as control of smooth muscle activity (Elphick and Melarange, 1998), olfaction (Müller and Hildebrandt, 1995), feeding (Colasanti *et al.*, 1995), and learning (Robertson *et al.*, 1996), are thought to be brought about by its activation of soluble guanylyl cyclase (sGC), which, by generating the intracellular second messenger cyclic GMP (cGMP), can control a variety of cellular functions. The NO-sGC-cGMP pathway has been demonstrated in a number of animal taxa, including invertebrates such as cnidarians (Colasanti *et al.*, 1995), annelids (Leake and Moroz, 1996), molluscs (Jacklet and

Koh, 1999), nematodes (Bascal *et al.*, 1995), arthropods (Müller, 1997), and echinoderms (Elphick and Melarange, 1998), as well as vertebrates (Ishii *et al.*, 1989). It remains to be seen if the NO-sGC-cGMP signaling pathway is involved in physiological responses of *A. pallida* to NO.

To our knowledge, the demonstration of NOS activity in the alga *Symbiodinium bermudense* is the first time that such activity has been described in symbiotic dinoflagellates. It is important to note that the activity induced in cultured zooxanthellae by incubation with a host-mimicking mixture of amino acids cannot be interpreted as activity emanating from residual anemone tissue that could possibly be present in incubations of zooxanthellae freshly isolated from a symbiotic relationship. It is clear that much more work is needed to fully characterize algal NOS activity before its functional significance can be understood with any confidence. If, however, as our bioassays indicate, NO is involved in the extension of this cnidarian's tentacles, it is tempting to speculate that, since the symbiotic algae are present in the highest densities in the anemone's tentacles, the NO that they generate may contribute to this behavior. Pearse (1974a, b) showed that zooxanthellate specimens of the anemone *Anthopleura elegantissima* expanded their tentacles in conditions of moderate light, and retracted them in bright light or in darkness, whereas azooxanthellate anemones were indifferent to light. Further experiments could test the hypothesis that symbiont-generated NO may be exerting a significant degree of control over the host's behavioral responses to light, an environmental factor of extreme importance to this photosynthesizing symbiotic assemblage.

In conclusion, we offer this characterization of NOS activity in the tropical marine anemone *A. pallida* as an addition to our knowledge of NOSs in invertebrates. Furthermore, the demonstration of NOS activity in the animal's symbionts, coupled with bioassays suggesting that NO may be involved in mediating behavioral aspects of the cnidarian-algal symbiotic assemblage, point towards a new role for the multifaceted signaling molecule NO. As we collect

and put into place more pieces of this puzzle, we believe that an interesting and attractive picture of the ways in which NO is involved in cnidarian-algal symbioses will be assembled.

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REFERENCES

- Bascal, Z. A., A. Montgomery, L. Holden-Dye, R. G. Williams, and R. J. Walker. 1995. Histochemical mapping of NADPH diaphorase in the nervous system of the parasitic nematode *Ascaris suum*. *Parasitology* 110:625-637.
- Bester, C., F. Lipschultz, R. Ream, D. Tomasino, and H. Trapido-Rosenthal. 1997. Effects of exposure to exogenous amino acids on the cellular physiology of cultured zooxanthellae. *Proc. 8th Internat. Coral Reef Symp.* II:1287-1290.
- Blachier, E., B. Darcy-Vrillon, A. Sener, P. Duee, and W. J. Malaisse. 1991. Arginine metabolism in rat enterocytes. *Biochim. Biophys. Acta* 1092:304-310.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Bredt, D. S. and S. H. Snyder. 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* 86:9030-9033.
- Bredt, D. S. and S. H. Snyder. 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.* 87:682-685.
- Bredt, D. S., P. M. Hwang, C. E. Glatt, C. Lowenstein, R. R. Reed, and S. H. Snyder. 1991. Cloned and expressed nitric oxide synthase closely resembles cytochrome P-450 reductase. *Nature* 351:714-718.
- Breer, H. and G. M. Shepherd. 1993. Implications of the NO/cGMP system for olfaction. *Trends Pharmacol. Sci.* 16:5-9.

- Burnett, A. L., C. J. Lowenstein, D. S. Bredt, T. S. K. Chang, and S. H. Snyder. 1992. Nitric oxide: A mediator of penile erection. *Science* 257:401–403.
- Colasanti, M., G. M. Lauro, and G. Venturini. 1995. NO in Hydra feeding response. *Nature* 374:505.
- Colasanti, M., G. Venturini, A. Merante, G. Musci, and G. Lauro. 1997. Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *J. Neurosci.* 17:493–499.
- Cook, C. B. 1983. Metabolic interchange in algae-invertebrate symbiosis. *Int. Rev. Cytol.* 14:117–210.
- Cook, C. B. and C. D. Orlandini. 1992. Initial studies of host factors inducing photosynthate release in corals (*Montastraea annularis*) and sea anemones (*Aiptasia pallida*). *Amer. Zool.* 32:111A.
- Elofsson, R., M. Carlberg, L. Moroz, L. Nezhlin, and D. Sakharov. 1993. Is nitric oxide produced in invertebrate neurons? *Neuroreport* 4:279–282.
- Elphick, M. R., I. C. Green, and M. O'Shea. 1993. Nitric oxide synthesis and action in the invertebrate brain. *Brain Research* 619:344–346.
- Elphick, M. R., D. G. Kemenes, K. Staras, and M. O'Shea. 1995a. Behavioural role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* 15:7653–7664.
- Elphick, M. R. and R. Melarange. 1998. Nitric oxide function in an echinoderm. *Biol. Bull.* 194:260–266.
- Elphick, M. R., R. C. Rayne, V. Riveros-Moreno, S. Moncada, and M. O'Shea. 1995b. Nitric oxide synthesis in locust olfactory interneurons. *J. Exp. Biol.* 198:821–829.
- Ferrier, M. D. 1992. Fluxes and metabolic pools of amino acids in algal-cnidarian symbioses. Ph.D. Diss., University of Maryland, College Park.
- Fitt, W. K. 1984. The role of chemosensory behavior of *Symbiodinium microadriaticum*, intermediate hosts, and host behavior in the infection of coelenterates and molluscs with zooxanthellae. *Mar. Biol.* 82:199–205.
- Fitt, W. K. 1985. Chemosensory responses of the symbiotic dinoflagellate *Symbiodinium microadriaticum* (Dinophyceae). *J. Phycol.* 21:62–67.
- Gates, R. D., O. Hoegh-Guldberg, M. J. McFall-Ngai, K. Y. Bil, and L. Muscatine. 1995. Free amino acids exhibit anthozoan "host factor" activity: They induce the release of photosynthate from symbiotic dinoflagellates *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 92:7430–7434.
- Giraldez, R. R. and J. L. Zweier. 1998. An improved assay for measurement of nitric oxide synthase activity in biological tissues. *Anal. Biochem.* 261:29–35.
- Griffiths, O. W. and S. S. Gross. 1996. Inhibitors of nitric oxide synthases. In M. Feelisch and J. S. Stamler (eds.), *Methods in nitric oxide research*, pp. 187–208. John Wiley & Sons Ltd, Chichester, UK.
- Guillard, R. R. J. 1975. Culture of phytoplankton for feeding marine invertebrates. In W. L. Smith and M. H. Chanley (eds.), *Culture of marine invertebrate animals*, pp. 29–60. Plenum Publishing Company, New York.
- Hughes, A. I. 1998. Protein phylogenies provide evidence of a radical discontinuity between arthropod and vertebrate immune systems. *Immunogenetics* 47:283–296.
- Ishii, K., L. D. Gorsky, Y. Förstermann, and F. Murad. 1989. Endothelium-derived releasing factor (EDRF): The endogenous activator of soluble guanylate cyclase in various types of cells. *J. Appl. Cardiol.* 4:505–512.
- Jacklet, J. W. and H.-Y. Koh. 1999. Nitric oxide stimulates cGMP production and mimics synaptic responses in metacerebral neurons of *Aplysia*. *J. Neurosci.* 10:3818–3826.
- Klatt, P., K. Schmidt, F. Brunner, and B. Mayer. 1994. Inhibitors of brain nitric oxide synthase: Binding-kinetics, metabolism and enzyme inactivation. *J. Biol. Chem.* 269:1674–1680.
- Korneev, S. A., J.-H. Park, and M. O'Shea. 1999. Neuronal expression of neural nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from an NOS pseudogene. *J. Neurosci.* 19:7711–7720.
- Korneev, S. A., M. R. Piper, J. Picot, R. Phillips, E. E. Korneeva, and M. O'Shea. 1998. Molecular characterization of NOS in a mollusc: Expression in a giant modulatory neuron. *J. Neurobiol.* 35:65–76.
- Leake, L. D. and L. L. Moroz. 1996. Putative nitric oxide synthase (NOS) containing cells in the central nervous system of the leech *Hirudo medicinalis*. *Brain Res.* 723:115–124.
- Liepe, A., C. Stone, J. Koistinaho, and D. R. Copenhagen. 1994. Nitric oxide synthase in Müller cells and neurons of salamander and fish retina. *J. Neurosci.* 14:7641–7654.
- Marietta, M. A. 1994. Nitric oxide synthase: Aspects concerning structure and catalysis. *Cell* 78:927–930.
- Martinez, A. 1995. Nitric oxide synthase in invertebrates. *Histochem. J.* 27:770–776.
- Masuda, K., S. Miyachi, and T. Maruyama. 1994. Sensitivity of zooxanthellae and non-symbiotic microalgae to stimulation of photosynthate excretion by giant clam tissue homogenate. *Mar. Biol.* 118:687–693.
- McCauley, P. J. 1986. Uptake of amino acids by cultured and freshly isolated symbiotic *Chlorella*. *New Phytol.* 104:415–427.
- McCauley, P. J. 1987. Quantitative estimation of movement of an amino acid from host to *Chlorella* symbionts in green hydra. *Biol. Bull.* 173:504–512.
- McCauley, P. J. 1988. Uptake of leucine by *Chlorella* symbionts of green hydra. *Proc. R. Soc. London B* 242:45–50.
- McCauley, P. J. 1991. Amino acids as a nitrogen source for *Chlorella* symbiotic with green hydra. *Hydrobiologica* 216/217:369–376.
- Morrall, C. E., H. G. Trapido-Rosenthal, A. H. Knap, and M. H. Depledge. 1998. Development of nitric oxide and nitric oxide synthase as ecotoxicological biomarkers in the tropical marine environment. *Marine Environ. Res.* 46:429–432.
- Morrall, C. E., T. S. Galloway, H. G. Trapido-Rosenthal, and M. H. Depledge. 2000. Characterisation

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- of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Comp. Biochem. Physiol. B* 125:483–491.
- Müller, U. 1997. The nitric oxide system in insects. *Prog. Neurobiol.* 51:368–381.
- Müller, U. and H. Hildebrandt. 1995. The nitric oxide/cGMP system in the antennal lobe of *Apis mellifera* is implicated in integrative processing of chemosensory stimuli. *Eur. J. Neurosci.* 7:2240–2248.
- Muscatine, L. 1967. Glycerol excretion by symbiotic algae from corals and *Tridacna*, and its control by the host. *Science* 156:516–519.
- Muscatine, L., R. R. Pool, and E. Cernichiaro. 1972. Some factors influencing selective release of soluble organic material by zooxanthellae from reef corals. *Mar. Biol.* 13:298–308.
- Muscatine, L. and J. W. Porter. 1977. Reef corals: Mutualistic symbioses adapted to nutrient-poor environments. *BioScience* 27:454–460.
- Nathan, C. and J. B. Hibbs. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* 3:65–70.
- Nathan, C. and Q.-W. Xie. 1994. Nitric oxide synthases: Roles, tolls and controls. *Cell* 78:915–918.
- Park, J.-H., V. A. Straub, and M. O'Shea. 1998. Anterograde signaling by nitric oxide: Characterization and *in vivo* reconstitution of an identified nitrergic synapse. *J. Neurosci.* 18:5463–5476.
- Pearse, V. B. 1974a. Modification of sea anemone behavior by symbiotic zooxanthellae: Phototaxis. *Biol. Bull.* 147:630–640.
- Pearse, V. B. 1974b. Modification of sea anemone behaviour by symbiotic zooxanthellae: Expansion and contraction. *Biol. Bull.* 147:641–651.
- Pollock, J. S., U. Forstermann, J. A. Mitchell, T. D. Warner, H. Schmidt, M. Nakane, and F. Murad. 1991. Purification and characterization of particulate endothelium derived relaxation factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 88:10480–10484.
- Radomski, W., J. F. Martin, and S. Moncada. 1991. Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (*Limulus polyphemus*). *Phil. Trans. R. Soc. London B* 334:129–133.
- Robertson, J. D., J. Bonaventura, A. Kohm, and M. Hiscat. 1996. Nitric oxide is necessary for visual learning in *Octopus vulgaris*. *Proc. Roy. Soc. London B* 263:1739–1743.
- Schmidt, H. H. H. S. and U. Walter. 1994. NO at work. *Cell* 78:99–925.
- Shick, J. M. 1991. A functional biology of sea anemones. Chapman and Hall, London.
- Stamler, J. S. 1994. Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* 78:931–936.
- Sutton, D. C. and O. Hoegh-Guldberg. 1990. Host-zooxanthella interactions in four temperate marine invertebrate symbioses: Assessment of effect of host extracts on symbionts. *Biol. Bull.* 178:175–186.
- Trench, R. K. 1971a. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. I. The assimilation of photosynthetic products of zooxanthellae by two marine coelenterates. *Proc. R. Soc. London B* 177:225–235.
- Trench, R. K. 1971b. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. II. Liberation of fixed ^{14}C by zooxanthellae *in vitro*. *Proc. R. Soc. London B* 177:237–250.
- Trench, R. K. 1971c. The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effect of homogenates of host tissues on the excretion of photosynthetic products *in vitro* by zooxanthellae from two marine coelenterates. *Proc. R. Soc. London B* 177:251–264.

Appendix 2

Details of tissue processing program applied to stained *A. pallida* after completion of the alcohol immersion sequence.

Equipment used: Shandon Hypercentre 2 (Program 2).

Step number	Reagent/ concentration	Temperature (°C)	Vacuum	Immersion	Drain
1		A	N	0	0.15
2		A	N	0	0.15
3	70% alcohol	A	N	14 h	0.15
4	90% alcohol	A	N	2 h	0.15
5	IMS	A	N	2 h	0.15
6	absolute alcohol	A	N	0.1 h	0.15
7	absolute alcohol	A	N	3.6 sec	0.15
8	xylene	A	N	1 h	0.15
9	xylene	A	N	30 min	0.15
10	xylene	A	N	16 min	0.15
11	wax 1	62	N	1 h	0.15
12	wax 2	60	Y	4 min	0.15

Key

IMS = 96% ethanol/ 3% methanol

A = ambient

REFERENCES

References

- Ahmad, S. *Antioxidant Mechanisms of Enzymes and Proteins*. In: Oxidative Stress and Antioxidant Defences in Biology. 238-272, 1997.
- Balser, E. and Fox, R.S. The role of the zooxanthellae *Symbiodinium microadriaticum* in the metabolism and behaviour of the sea anemone *Aiptasia pallida*. *American Zoologist*. 274A, 1998.
- Banaszak, A.T. and Trench, R.K. Effects of ultraviolet (UV) radiation on marine microalgal-invertebrate symbioses. II. The synthesis of mycosporine-like amino acids in response to exposure to UV in *Anthopleura elegantissima* and *Cassiopeia xamachana*. *J. Exp. Marine Biology and Ecology* 194: 233-250, 1995.
- Bargmann, C.I. Neurobiology of the *Caenorhabditis elegans* Genome. *Science* 282: 2028-2033, 1998.
- Bascal, Z. A., Montgomery, A., Holden-Dye, L., Williams, R. G., and Walker, R. J. Histochemical mapping of NADPH diaphorase in the nervous system of the parasitic nematode *Ascaris suum*. *Parasitology*. 110:625-637, 1995
- Bastidas, C., Bone, D., and Garcia, E.M. Sediment Rates and Metal content of sediments in a Venezuelan coral reef. *Marine Pollution Bulletin*. 38 (1): 16-24, 1999.
- Batham, E.J. and Pantin, C.F.A. Inherent activity in the sea anemone *Metridium senile*. *J. Exp. Biology* 27 (3/4): 290-301, 1950b.
- Batham, E.J. and Pantin, C.F.A. Muscular and hydrostatic action in the sea anemone *Metridium senile*. *J. Exp. Biology*. 27: 264-289, 1950c.
- Batham, E.J. and Pantin, C.F.A. Phases of activity in the sea anemone *Metridium senile* and their relation to external stimuli. *J. Exp. Biology* 27 (3/4): 377-399, 1950a.
- Batham, E.J. and Pantin, C.F.A. Slow contraction and its relation to spontaneous activity in the sea anemone *Metridium senile*. *J. Exp. Biology*. 84-103, 1953.

References

- Beaumont, A.R. and Newman, P.B. Low levels of tributyl tin reduce growth of marine micro-algae. *Mar. Poll. Bull.* 17 (10): 457-461, 1986.
- Beck, K.F., Eberhardt, W., Frank, S., Huwiler, A., Mebmer, U.K., and Pfeilschifter, J. Inducible NO synthase: Role in cellular signalling. *Journal Experiment. Biol.* 202: 645-653, 1999.
- Belda-Baillie, C.A., Leggat, W., and Yellowlees, D. Growth and metabolic responses of the giant clam zooxanthellae symbiosis in a reef-fertilization experiment. *Marine Ecology Progress Series* 170: 131-141, 1998.
- Benazet-Tambutte, S. and Allemand, D. Water permeability of the oral epithelial layers of the sea anemone *Anemonia viridis*. *J. Experiment. Zool.* 279: 1-8, 1997.
- Benzie, I.F.F. and Strain, J.J. Ferric reducing/ antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods In Enzymology* 299: 15-27, 1999.
- Benzie, I.F.F. and Strain, J.J. Simultaneous automated measurement of total 'antioxidant' (reducing) capacity and ascorbic acid concentration. *Redox Report* 3 (4): 233-238, 1997.
- Benzie, I.F.F. and Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': The FRAP assay. *Analytical Biochemistry* 239:70-76, 1996.
- Blachier, F., Darcy-Vrillon, B., Sener, A., Duee, P., and Malaisse, W. Arginine metabolism in rat erythrocytes. *Biochim. Biophys. Acta.* 1092: 304-310, 1991.
- Black, N.A., Voellmy, R., and Szmant, A.M. Heat shock protein induction in *Montastrea faveolata* and *Aiptasia pallida* exposed to elevated temperatures. *Biological Bulletin.* 188: 234-240, 1995.
- Blaise, C. and Kusui, T. Acute toxicity assessment of industrial effluents with a microplate-based *Hydra attenuata* assay. *Environmental Toxicology and Water Quality* 12 (1), 1997.

References

- Bosch, T.C.G., Krylow, S.M., Bode, H.R., and Steele, R.E. Thermotolerance and synthesis of heat shock proteins: these responses are present in *Hydra attenuata* but absent in *Hydra oligactis*. *Proc. Natl. Acad. Sci.* 85: 7927-7931, 1988.
- Bouchard, N., Pelletier, E., and Fournier, M. Effects of butyltin compounds on phagocytic activity of haemocytes from three marine bivalves. *Environmental Toxicology and Chemistry*. 18 (3): 519-522, 1999.
- Bradford, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principal of protein-dye binding. *Analytical Biochemistry*. 72: 248-254, 1976.
- Branton, M.A., MacRae, T.H., Lipschultz, F., and Wells, P.G. Identification of a small heat shock/alpha-crystallin protein in the scleractinian coral *Madracis mirabilis* (Duch. and Mitch.). *Can. J. Zool.* 77: 675-682, 1999.
- Bredt, D. S. and Snyder, S. H. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci.* 86: 9030-9033, 1989.
- Bredt, D.S. and Schmidt, H.H.H.W. *The citrulline assay*. In: Methods in NO Research. Eds. Feelisch, M. and Stamler, J.S. Wiley. 249-255, 1997.
- Bredt, D.S. and Snyder, S.H. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci.* 86: 9030-9033, 1989.
- Breer, H. and Shepard, G. M. Implications of the NO/cGMP system in olfaction. *Trends in Neuroscience*. 16: 5-8, 1993.
- Brown, B. Coral Bleaching: Causes and consequences. *Proc 8th Int Coral Reef Symp* 1: 65-74, 1997.
- Brown, B.E. and Howard, L.S. Assessing the effects of 'stress' on reef corals. *Advances in Marine Biology*. 22:1-63, 1985.

References

- Brown, B.E., Le Tissier, M.D.A., and Bythell, J.C. Mechanisms of bleaching deduced from histological studies of reef corals sampled during a natural bleaching event. *Marine Biology* 122: 655-663, 1995.
- Brown, G.C. Nitric oxide and mitochondrial respiration. *Biochimica et Biophysica Acta* 1411: 351-369, 1999.
- Bryan, G.W. and Gibbs, P.E. Heavy metals in the Fal estuary, Cornwall: a study of long-term contamination by mining waste and its effects on estuarine organisms. *Occ. Publs Mar. Biol. Ass. U.K.* 2: 2-112, 1983.
- Bryant, D., Burke, L., McManus, J., and Spalding, M. Reefs at Risk. A map-based indicator of the threats to the world's coral reefs. *Reefs at Risk* 1 (1): 1-56, 1998.
- Buddemeier, R.W. and Fautin, D.G. Coral bleaching as an adaptive mechanism a testable hypothesis. *Bioscience* 43 (5): 320-325, 1993.
- Buddemeier, R.W. Making light work of adaptation. *Nature* 388: 229-230, 1997.
- Burdon, R.H., Gill, V. and Rice-Evans. Active oxygen and heat shock protein induction. In: *Stress Proteins* (19-25). Eds. Schlesinger, M.G., Santoro, M.G. and Garaci. Springer-Verlag, Heidelberg, 1990.
- Bythell, J.C., Sharp, V.A., Miller, D., and Brown, B.E. A novel environmentally regulated 33 kDa protein from tropical and temperate cnidarian zooxanthellae. *J Them. Biol.* 20 (1/2): 15-22, 1995.
- Cao, G., Alessio, H.M. and Cutler, R.G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Rad. Biol. Med.* 14: 303-311, 1993.
- Cao, G. and Prior, R.L. Comparison of different analytical methods of assessing total antioxidant capacity of human serum. *Clinical Chemistry.* 44 (6): 1309-1315, 1998.

References

- Cardwell, R.D., Brancato, M.S., Toll, J., DeForest, D., and Tear, L. Aquatic ecological risks posed by Tributyltin in United States surface waters: pre-1989 to 1996 data. *Environmental Toxicology and Chemistry*. 18 (3): 567-577, 1999.
- Cavaliere, A. R., Barnes, R.D. and Cook, C.B. Field Guide to the Conspicuous Flora and Fauna of Bermuda. 2nd edition. BBSR special publication. 1987.
- Chen, Y. and Rosazza, J. P. A bacterial nitric oxide synthase from a *Nocardia* species *Biochem. Biophys. Res. Commun.* 203:1251-1258, 1994.
- Cheng, Y.C. and Prusoff, W.H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes a 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochemical Pharmacology*. 22: 3099-3108, 1973.
- Christie, N. and Costa, M. Review: In vitro assessment of the toxicity of metal compounds. IV. Disposition of metals in cells: interaction with membranes, glutathione, metallothioneins and DNA. *Biol. Trace. Elem. Res.* 6: 139-158, 1984.
- Cima, F., Ballarin, L., Bressa, G., and Sabbadin, A. Immunotoxicity of butyltins in tunicates. *Applied Organometallic Chemistry* 9:567-572, 1995.
- Clark, K.B. and Jensen, K.R. Effects of temperature on carbon fixation and carbon budget partitioning in the zooxanthellal symbiosis of *Aiptasia pallida* (Verill). *J. Exp. Mar. Biol. and Ecol.* 64:215-230, 1982.
- Clayton, W.S. and Lasker, H.R. Host feeding regime and zooxanthellae photosynthesis in the anemone *Aiptasia pallida* (Verill). *Biol. Bull.* 167:590-600, 1984.
- Clayton, W.S. and Lasker, H.R. Individual and population growth in the asexually reproducing anemone *Aiptasia pallida* (Verill). *J. Exp. Mar. Biol. and Ecol.* 90: 249-258, 1985.
- Clayton, W.S. Factors affecting zooplankton feeding by the sea anemone *Aiptasia pallida*. *Helgolander Meeresuntersuchungen* 40: 83-90, 1986b.

References

- Clayton, W.S. Ingestion, digestion and assimilation efficiency of the sea anemone *Aiptasia pallida* fed zooplankton prey. *Int. Revue ges Hydrobiol* 71 (5): 709-717, 1986a.
- Clayton, W.S. Pedal laceration by the anemone *Aiptasia pallida*. *Marine Ecology Progress Series* 21:75-80, 1985.
- Colasanti, M. and Venturini, G. Nitric Oxide in Invertebrates. *Molecular Neurobiology* 17: 157-174, 1998.
- Colasanti, M., Lauro, G. M. and Venturini, G. NO in Hydra feeding response. *Nature*. 374: 505, 1995.
- Colasanti, M., Venturini, G., Merante, A., Musci, G., and Lauro, G.M. Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *Journal of Neuroscience* 17 (1): 493-499, 1997.
- Coles, S.L. and Jokiel, P.L. Effects of temperature on photosynthesis and respiration in hermatypic corals. *Marine Biology* 43: 209-216, 1977.
- Conte, A. and Ottaviani, E. Nitric Oxide synthase activity in molluscan haemocytes. *FEBS Lett.* 365: 120-124, 1995.
- Cook, C.B., Logan, A., Ward, J., Luckhurst, B., and Berg, C.J. Elevated temperatures and bleaching on a high latitude coral reef: the 1988 Bermuda Event. *Coral Reefs* 9: 45-49, 1990.
- Cook, C.B., Muller-Parker, G., and D'Elia, C.F. Ammonium enhancement of dark carbon fixation and nitrogen limitation in symbiotic zooxanthellae: effects of feeding and starvation of the sea anemone *Aiptasia pallida*. *Limnology. Oceanogr.* 37 (1): 131-139, 1992.
- Cook, C.B., Muller-Parker, G., and D'Elia, C.F. Host feeding and nutrient sufficiency for zooxanthellae in the sea anemone *Aiptasia pallida*. *Marine Biology* 98: 253-262, 1988.

References

- Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R., Paruelo, J., Raskin, R.G., Sutton, P., and van den Belt, M. The value of the world's ecosystem services and natural capital. *Nature* 387: 253-260, 1997.
- Crane, B. R., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science*. 278: 425-431, 1997
- Davies, K.J.A. Oxidative stress: the paradox of aerobic life. Edited by Rice-Evans, C. and Halliwell, B. Portland Press. 1-31, 1994.
- Dawson, T. M. and Snyder, S.H. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J. Neurosci.* 14: 5147-5159, 1994.
- Dawson, T.M., Brecht, D.S., Fotuhi, M., Hwang, P.M., and Snyder, S.H. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci.* 88: 7797-7801, 1991.
- Delledonne, M., Xia, Y., Dixon, R. A., Lamb, C. Nitric oxide functions as a signal in plant disease resistance. *Nature*. 394: 585-588. 1998.
- Denton, G.R.W. and Burdon-Jones, C. Trace metals in surface water from the Great Barrier Reef. *Marine Pollution Bulletin* 17 (3): 96-98, 1986.
- Depledge, M.H. Ecotoxicology in Theory and Practice. Series forward, vii-ix. Ed. Forbes, V.E. and Forbes, T.L. Chapman and Hall. 1994
- Depledge, M.H. New approaches in ecotoxicology; can inter-individual physiological variability be used as a tool to investigate pollution effects. *AMBIO* 19 : 251-252, 1990.
- Depledge, M.H. The rational basis for detection of the early effects of marine pollutants using physiological indicators. *Ambio*. 18 (5): 301-302. 1989.

References

- Depledge, M.H. The rational basis for the use of biomarkers as ecotoxicological tools. 271-295. In: *Nondestructive Biomarkers in Vertebrates*. Ed. Fossi, M.C. and Leonzio, C. Lewis Publishers, Inc. 1993.
- Depledge, M.H., Aagaard, A. and Gyorkos, P. Assessment of trace metal toxicity using molecular, physiological and behavioural biomarkers. *Mar. Poll. Bull.* 31 (1-3): 19-27, 1995
- Depledge, M.H. and Billingham, Z. Ecological significance of endocrine disruption in marine invertebrates. *Mar. Poll. Bull.* 39 (1-12): 32-38, 1999.
- Depledge, M.H. and Fossi, M.C. The role of biomarkers in environmental assessment (2). Invertebrates. *Ecotoxicology*. 3: 161-172, 1994.
- Depledge, M.H. and Rainbow, P.S. Models of regulation and accumulation of trace metals in marine invertebrates. *Comp. Biochem. and Physiol.* 97C: 1-7, 1990.
- Depledge, M.H., Weeks J.M. and Bjerregaard P. Heavy Metals. 79-105. In: *Handbook of Ecotoxicology*, vol. 2. Ed. P. Calow. Blackwell Publ., Cambridge. 1994.
- Dodge, E.R., Logan, A., and Antonius, A. Quantitative reef assessment studies in Bermuda: a comparison of methods and preliminary results. *Bulletin of Marine Science* 32 (3): 745-760, 1982.
- Dodge, R.E. and Brass, G.W. Skeletal extension, density and calcification of the reef coral, *Montastrea annularis*: St Croix, US. Virgin Islands. *Bulletin of Marine Science* 34 (2): 288-307, 1984.
- Dodge, R.E., Wyers, S.C., Frith, H.R., Knap, A.H., Smith, S.R., Cook, C.B., and Sleeter, T.D. Coral calcification rates by the buoyant weight technique: effects of Alizarin staining. *J. Exp. Mar. Bio. Ecol.* 75: 217-232, 1984.
- Donkin, P., Widdows, J., Evans, S.V., and Brinsley, M.D. QSARS's for the sublethal responses of marine mussels (*Mytilus edulis*). *The Science of the Total Environment* 109/110: 461-476, 1991.

References

- Douglas, A.E. *Symbiosis as a source of novel metabolic capabilities*. In: Symbiotic Interactions. Oxford University Press. 12-38, 1984.
- Downs, C.A., Muller, E., Phillips, S., Fauth, J.E. and Woodley, C.M. A molecular biomarker system for assessing the health of coral (*Montastrea faveolata*) during heat stress. *Marine Biotechnology*. 2 (6): 533-544, 2000.
- Duquesne, S.J. and Coll, J.C. Metal Accumulation in the clam *Tridacna crocea* under natural and experimental conditions. *Aquatic Toxicology*. 32: 239-253, 1995.
- Dyken, J.A. and Shick, J.M. Oxygen production by endosymbiotic algae controls superoxide dismutase activity in their animal host. *Nature* 297: 579-580, 1982.
- Dyken, J.A. and Shick, J.M. Photobiology of the symbiotic sea anemone, *Anthopleura elegantissima*: defences against photodynamic effects, and seasonal photoacclimatization. *Biol. Bull.* 167: 683-697, 1994.
- Dyken, J.A., Shick, J.M., Benoit, C., Buettner, G.R., and Winston, G.W. Oxygen radical production in the sea anemone *Anthopleura elegantissima* and its symbiotic algae. *J. Exp. Biol.* 168: 219-241, 1992.
- Edinger, E.N., Jompa, J., Limmon, G.V., Widjatmoko, W., and Risk, M.J. Reef degradation and coral biodiversity in Indonesia. Effects of land-based pollution, destructive fishing practices and changes over time. *Marine Pollution Bulletin* 36 (8): 617-630, 1998.
- Edmunds, M., Potts, G.W., Swinfen, R.C., and Waters, V.L. Defensive behaviour of sea anemones in response to predation by the opisthobranch mollusc *Aeolidia papillose*. *JMBA* 56: 65-83, 1976.
- Eertman, R.H.M., Groenink, C.L.F.M.G., Sandee, B., and Hummel, H. Response of the blue mussel *Mytilus edulis* following exposure to PAH's or contaminated sediment. *Marine Environmental Research*. 39: 169-173, 1995.

References

- Elofsson, R., Carlberg, M., Moroz, L., Nezhlin, L. and Sakharov, D. Is nitric oxide produced in invertebrate neurons? *Neuroreport*. 4: 279-282, 1993.
- Elphick, M. R. and Melarange, R. Nitric oxide function in an echinoderm. *Biol. Bull.* 194:260-266, 1998.
- Elphick, M. R., Kemenes, G., Staras, K. and O'Shea, M. Behavioural role for nitric oxide in chemosensory activation of feeding in a mollusc. *J. Neurosci.* 15: 7653-7664, 1995.
- Elphick, M.R., Green, I.C., and O'Shea, M. Nitric oxide synthesis and action in an invertebrate brain. *Brain Research*. 619: 344-346, 1993.
- Engebretson, H. and Martin, K.M. Effects of decreased salinity on expulsion of zooxanthellae in the symbiotic sea anemone *Anthopleura elegantissima*. *Pacific Science* 48 (4): 446-457, 1994.
- Evans, E. C. Helgolander Wiss Meeresunters, 30: 178-191, 1977.
- Evans, S.M. Tributyltin pollution: the catastrophe that never happened. *Marine Pollution Bulletin*. 38 (8): 629-636, 1999.
- Evans, S.M., Leksono, T., and McKinnell, P.D. Tributyltin pollution: a diminishing problem following legislation limiting use of the TBT based anti-fouling paints. *Mar. Poll. Bull.* 30 (1): 14-21, 1995.
- Fagoonee, I., Wilson, H.B., Hassell, M.P., and Turner, J.R. The dynamics of zooxanthellae populations: a long-term study in the field. *Science*. 283: 843-845, 1999.
- Feelisch, M. and Stamler, J. S. *Donors of Nitric Oxides*. In: Methods in NO research. Eds. Feelisch, M. and Stamler, J.S. Wiley. 71-115. 1997a.
- Feelisch, M. and Stamler, J.S. *Measurement of NO-related activities-which assay for which purpose?* In: Methods in NO research. Eds. Feelisch, M. and Stamler, J.S. Wiley. 302-307, 1997b.

References

- Felton, G.W. *Oxidative stress of vertebrates and invertebrates*. In: *Oxidative stress and antioxidant defences in biology*. Ed. Ahmad, S. 356-434, 1995.
- Fent, K. Ecotoxicology of organotin compounds. *Critical Reviews in Toxicology* 26 (1): 1-117, 1996.
- Ferrier, M.D. Fluxes and metabolic pools of amino acids in algal-cnidarian symbioses. University of Maryland. PhD thesis. 1-177, 1992.
- Fish, R.H., Kimmell, E.C., Casida, J.E. Bioorganotin chemistry: reactions of tributyltin derivatives with cytochrome P-450 dependent monooxygenase enzyme system. *J. Organomet. Chem.* 118: 41-54, 1976.
- Fitt, W.K. and Warner, M.E. Bleaching patterns of four species of Caribbean reef corals. *Biol. Bull.* 189: 298-307, 1995.
- Forbes, V.E., Forbes, T.L., and Holmer, M. Inducible metabolism of fluoranthene by the opportunistic polychaete *Capitalla* sp. I. *Marine Ecology Progress Series* 132: 63-70, 1996.
- Franchini, A., Conte, A., and Ottaviani, E. Nitric Oxide: An ancestral immunocyte effector molecule. *Advances in Neuroimmunology*. 5: 463-478, 1995a.
- Franchini, A., Fontanili, P., and Ottaviani, E. Invertebrate immunocytes: relationship between phagocytosis and nitric oxide production. *Comp. Biochem. Physiol* 110B (2): 403-407, 1995b.
- Friese, U.E. *Sea Anemones*, T.F.H. Publications. 1-128. 1972.
- Galloway, T. S. and Depledge, M. H. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology*. 10: 5-23, 2001.
- Gamalay, I.A. and Klyubin, I.V. Roles of reactive oxygen species: signalling and regulation of cellular functions. *International Review of Cytology*. 188: 203-255, 1999.

References

- Gardella, D.J. and Edmunds, P.J. The oxygen microenvironment adjacent to the tissue of the scleractinian *Dichocoenia stokesii* and its effects on symbiont metabolism. *Marine Biology*. 135: 289-295, 1999.
- Garthwaite, J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends in Neuroscience*. 14: 60-67, 1991.
- Gates, R.D., Baghaadasarian, G., and Muscatine, L. Temperature stress causes host cell detachment in symbiotic cnidarians: implications for coral bleaching. *Biol. Bull* 182: 324-332, 1992.
- Global Coral Reef Monitoring Network (GCRMN). *Status of the coral reefs of the world: 2000*. Ed. Wilkinson, C. Australian Institute Marine Science, Townsville, Australia. 1-7 (summary), 2002.
- Giovanelli, J., Campos, K.L., and Kaufman, S. Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of Arginine. *Proc. Natl. Acad. Sci. USA*. 88: 7091-7095, 1991.
- Giraldez, R. R. and Zweier. J. L. An improved assay for measurement of nitric oxide synthase activity in biological tissues. *Analytical. Biochemistry*. 261: 29-35, 1998.
- Glider, W.V., Phipps, D.W., and Pardy, R.L. Localisation of symbiotic dinoflagellate cells within tentacle tissue of *Aiptasia pallida*. *Trans. Amer. Micro. Soc.* 99 (4): 426-438, 1980.
- Glynn, P.W. and D'Croz, L.D. Experimental evidence for high temperature stress as the cause of El Nino-coincident coral mortality. *Coral Reefs*. 8: 181-191, 1990.
- Glynn, P.W. Coral Reef bleaching: ecological perspectives. *Coral Reefs*. 12: 1-17, 1993a.
- Glynn, P.W. Extensive bleaching and death of reef corals on the pacific coast of Panama. *Environmental Conservation*. 10 (2): 149-154, 1983b.

References

- Glynn, P.W. Widespread coral mortality and the 1982-83 El Nino Warming event. *Environmental Conservation*. 11 (2): 133-146, 1984.
- Glynn, P.W., Howard, L.S., Corcoran, E.F., and Freay, A.D. The occurrence and toxicity of herbicides in reef building corals. *Marine Pollution Bulletin*. 15 (10): 370-374, 1984.
- Glynn, P.W., Perez, M., and Gilchrist, S.L. Lipid decline in stressed corals and their crustacean symbionts. *Biol. Bull.* 168: 276-284, 1985a.
- Glynn, P.W., Peters, E.C., and Muscatine, L. Coral tissue microstructure and necrosis: relation to catastrophic mortality in Panama. *Diseases of aquatic organisms* 1: 29-37, 1985b.
- Goksoyr, A., Larsen, H.L., and Husoy, A.M. Application of a cytochrome P-450 IA1-ELISA in environment monitoring and toxicological testing of fish. *Comparative Biochemistry and Physiology*. 100C (1/2): 157-160, 1991.
- Goreau, T.J. and Macfarlane, A.H. Reduced growth rate of *Montastrea annularis* following the 1987-1988 coral bleaching event. *Coral Reefs* 8: 211-215, 1990.
- Gorian, C., Allemand, D., and Galgani, I. Transient Na stress in symbiotic dinoflagellates after isolation from coral-host cells and subsequent immersion in seawater. *Marine Biology* 129: 581-589, 1997.
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., and Tannenbaum, S.R. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Analytical biochemistry*. 126: 131-138, 1982.
- Griffith, O. W. and Gross, S. S. *Inhibitors of Nitric Oxide Synthases*. Methods in Nitric Oxide Research. Eds. Feelisch, M. and Stamler, J. S. John Wiley & Sons Ltd, Chichester, UK. 187-208, 1996.
- Griffith, O. W. and Stuehr, D. J. Nitric oxide synthases. Properties and catalytic mechanisms. *Annu. Rev. Physiol.*; 57:707-736, 1995.

References

- Grigg, R.W. Effects of sewage discharge, fishing pressure and habitat complexity on coral ecosystems and reef fishes in Hawaii. *Marine Ecology Progress Series*. 103: 25-34, 1994.
- Griscavage, J.M., Fukuto, J.M., Komori, Y., and Ignarro, L.J. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with heme prosthetic group. *Journal of Biological Chemistry* 269 (34): 21644-21649, 1994.
- Grundy, M.M., Moore, M.N., Howell, S.M., and Ratcliffe, N.A. Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology*. 34:273-290, 1996.
- Guzman, H.M. and Jimenez, C. Contamination of coral reefs by heavy metals along the Caribbean coast of central America (Costa Rica and Panama). *Marine Pollution Bulletin* 24 (11): 554-561, 1992.
- Halliwell, B. and Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*. 219: 1-14, 1984
- Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *The Lancet*. 344: 721-724, 1994a.
- Halliwell, B. *How to characterize an antioxidant: an update*. In: Free Radicals and Oxidative Stress: Environmental, Drugs and Food Additives. Eds. Rice-Evans, C. and Halliwell, B. Portland Press. 73-101, 1994b.
- Hannack, K., Kestler, P., Sicken, O., and Westheide, W. The influence of UV radiation on number and ultrastructure of endosymbiotic dinoflagellates in the sea anemone *Ceruss pedunculatus* (Anthozoa: Actinaria). *Hegolander meeresuntersuchungen*. 51: 487-502, 1997.
- Hardy, J.T., Hoge, F.E., Yungel, J.K., and Dodge, R.E. Remote detection of coral bleaching using pulsed-laser fluorescence spectroscopy. *Marine Ecology Progress Series* 88: 247-255, 1992.

References

- Harland, A.D. and Brown, B. E. Metal tolerance in the scleractinian coral *Porites lutea*. *Marine Pollution Bulletin*. 20 (7): 353-357, 1989.
- Harland, A.D. and Nganro, N.R. Copper uptake by the sea anemone *Anemonia viridis* and the role of zooxanthellae in metal regulation. *Marine Biology*. 104: 297-301, 1990.
- Harland, A.D., Bryan, G.W., and Brown, B.E. Zinc and Cadmium absorption in the symbiotic anemone *Anemonia viridis* and the non-symbiotic anemone *Actinia equina*. *JMBA*. 70: 789-802, 1990.
- Harriott, V.J. Coral lipids and environmental stress. *Environmental monitoring and Assessment* 25: 131-139, 1993.
- Hatcher, B.G., Johannes, R.E., and Robertson, A.I. Review of Research relevant to the conservation of shallow tropical marine ecosystems. *Oceanography Marine Biol. Annu. Rev.* 27: 337-414, 1989.
- Hay, R.W. Bio-Inorganic Chemistry. Ellis Horwood, Camelot Press, Great Britain. 1984.
- Hayes, R.L. and Bush, P.G. Microscopic observations of recovery in the reef-building scleractinian coral, *Montastrea annularis*, after bleaching on a Cayman reef. *Coral Reefs* 8: 203-209, 1990.
- Hayes, R.L. and King, C.M. Induction of 70-kD heat shock protein in scleractinian corals by elevated temperature: significance for coral bleaching. *Molecular Marine Biology and Biotechnology* 4 (1): 36-42, 1995.
- Hecker, M. and Billiar, T.R. *Detection of L-arginine, related metabolites and guanidini compounds*. In: Methods in Nitric Oxide Research. Eds. Feelisch, M. and Stamler, J.S. John Wiley and Sons, Ltd. 259-270. 1996.

References

- Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E. and Varic, J. R. The metabolism of L-arginine and its significance for the biosynthesis of endothelium derived releasing factor. Cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. USA*. 87: 8612-8616, 1990.
- Hey, C., Boucher, J.L., Vadou-Le Goff, S., Ketterer, G., Wessler, I., and Racje, K. *Br. J. Pharmacol.* 121, 395-400, 1997.
- Hibbs, J.B. Jr, Taintor, R.R., Vavrin, Z., Granger, D.L., Drapier, D.C., Amber, I.J. and Lancaster, J.R., Jr. *Synthesis of nitric oxide from a terminal guanidino nitrogen atom L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron*. 189-223. In: Nitric Oxide from L-arginine: A Bioregulatory System. Eds. Moncada, S. and Higgs, E.A. Elsevier, Amsterdam. 1990.
- Hidalgo, E., Ding, H., and Demple, B. Redox signal transduction via iron-sulfur clusters in the SoxR transcription activator. *TIBS*. 22: 207-210, 1997.
- Higuchi, H., Granger, D.N., Saito, H., and Kurose, I. Assay of antioxidant and anti-inflammatory activity of nitric oxide *in vivo*. *Methods In Enzymology*. 301: 424-435, 1999.
- Hoegh-Guldberg, O. Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshwater res.* 50: 839-866, 1999.
- Hoegh-Guldberg, O. and Smith, G.J. The effect of sudden changes in temperature, light and salinity on the population density and export of zooxanthellae from the reef corals *Stylophora pistillata* (Esper) and *Seriatopora hystrix* (Dana). *J. Exp. Mar. Biol. and Ecol.* 129: 279-303, 1989.
- Hoegh-Guldberg, O. McCloskey, L.R. and Muscatine, L. Expulsion of zooxanthellae by symbiotic cnidarians from the Red Sea. *Coral Reefs*. 5: 201-204, 1987.
- Hope, B.T., Michael, K.M., Knigge, K.M., and Vincent, S.R. Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc. Natl. Acad. Sci.* 88: 2811-2814, 1991.

References

- Howard, L.S. and Brown, B.E. Heavy metals and reef corals. *Oceanogr. Mar. Biol. Ann. Rev.* 22: 195-210, 1984.
- Huang, S.P., Lin, K.L., and Fang, L.S. The involvement of calcium in heat-induced coral bleaching. *Zoological studies*. 37 (2): 89-94, 1998.
- Hughes, A. L. Protein phylogenies provide evidence of a radical discontinuity between arthropod and vertebrate immune systems. *Immunogenetics*. 47: 283-296, 1998.
- Hughes, T.P. and Connell, J.H. Multiple stressors on coral reefs: a long-term perspective. *Limnol. Oceanogr.* 44 (3, part 2): 932-940, 1999.
- Hunter, T. The energetics of asexual reproduction: pedal laceration in the symbiotic sea anemone *Aiptasia pulchella* (Carlgreen 1943). *J. Exp. Mar. Biol. Ecol.* 83: 127-147, 1994.
- Hyman, L.H. *Metazoa of the tissue grade of construction-the radiate phyla- phylum Cnidaria*. In: The Invertebrates Protozoa Through to Ctenophora. Volume 1. Ed. Boell, E J. McGraw-Hill Book Company, Inc. 365-661, 1940.
- Iadecola, C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends in Neuroscience* 20 (3): 132-139, 1997.
- Iglesias-Prieto, R., Matta, J.L., Robins, W.A., and Trench, R.K. Photosynthetic response to elevated temperature in the symbiotic dinoflagellate *Symbiodinium microadriaticum* in culture. *Proc. Natl. Acad. Sci. USA*. 89: 10302-10305, 1992.
- Ignarro, L. J., Buga, G. M., Wood, K. S., Byrnes, R. E. and Chavdhuri, G. Endothelium derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA*. 84: 9265-9269, 1987.
- Ignarro, L. J., Buga, G.M., Wood, K.S., Byrnes, R.E. and Chanudhuri, G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA*. 84: 9265-9269, 1987.

References

- Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B. and Bloch, K. D. Cloning and expression of a cDNA-encoding human endothelial-derived relaxing factor nitric oxide synthase. *J. Biol. Chem.* 267: 14519-14522, 1992.
- Jeffery, S.W. and Humphrey, G.F. New spectrophotometric equations for determining chlorophyll's a, b c and c₂ in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflazen.* 167: 191-194, 1975.
- Jia, L., Bonaventura, J., and Stamler, J.S. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* 380: 221-226, 1996.
- Johansson, K.U.I. and Carlberg, M. NO-synthase: what can research in invertebrates add to what is already known? *Advances in Neuroimmunology.* 5: 431-442, 1995.
- Jones, P. Florida's Coral in Decline. *Marine Pollution Bulletin.* 24 (3): 127, 1995.
- Jones, R.J. and Hoegh-Guldberg, O. Effects of cyanide on coral photosynthesis: implications for identifying the cause of coral bleaching and for assessing the environmental effects of cyanide fishing. *Mar. Ecol. Prog. Ser.* 177:83-91, 1999.
- Jones, R.J. Zooxanthellae loss as a bioassay for assessing stress in corals. *Marine Ecology Progress Series* 149: 163-171, 1997.
- Jones, R.J., Hoegh-Guldberg, O., Larkham, A.W.D., and Schreiber, U. Temperature-induced bleaching of corals begins with impairment of the CO₂ fixation mechanism in zooxanthellae. *Plant, Cell and Environment.* 21: 1219-1230, 1998.
- Kasschau, M.R., Skaggs, M.M. and Chen, E.C.M. Accumulation of glutamate in sea anemones exposed to heavy metals and organic amines. *Bull. Environm. Contam. and Toxicol.* 25, 873-878. 1980.
- Kelly, S.A., Havrilla, C.M., Brady, T.C., Abramo, K.H., and Levin, E.D. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. *Environmental Health Perspectives* 106 (7): 375-384, 1998.

References

- Kergosien, D. and Rice, C.D. Macrophage secretory function is enhanced by low doses of tributyltin oxide (TBTO), but not tributyltin chloride (TBTCl). *Archives of Environmental Contamination and Toxicology* 34: 223-228, 1998.
- Kiechle, F.L. and Malinski, T. Nitric oxide: Biochemistry, pathophysiology and detection. *A.J.C.P.* 100 (5): 567-575, 1993.
- Kinsey, D.W. Coral reef system response to some natural and anthropogenic stresses. *Galaxea*. 7: 113-128, 1988.
- Kizner, Z., Achituv, Y., Dubinsky, Z., and Thielberger, Y. A novel, non-destructive bioassay for assessing a real chlorophyll a in hermatypic cnidarians. *Limnol. Oceanogr.* 40 (6): 1166-1173, 1995.
- Klatt, P., Schmidt, K., Brunner, F. and Mayer, B. Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism, and enzyme inactivation. *J. Biol. Chem.* 269, 1674-1680. 1994.
- Knowles, R. G. and Moncada, S. Nitric oxide as a signal in blood vessels. *Trends Biochem. Sci.* 17: 399-402, 1992.
- Knowles, R.G. and Moncada, S. Nitric Oxide Synthases in mammals. *Biochemical Journal.* 298: 249-258, 1994.
- Kolb, H. and Kolb-Bachofen, V. Nitric oxide: a pathogenic factor in autoimmunity. *Immunology Today.* 13 (5): 157-160, 1992.
- Krone, M. A. and Biggs, D.B. Research on environmental fate and effects of drilling muds and cuttings (symposium proceedings). Lake Buena Vista, Florida. 2: 1079-1101, 1980.
- Kuhlmann, D.H.H. The sensitivity of coral reefs to environmental pollution. *AMBIO.* 17 (1): 13-21, 1988.

References

- Kushmaro, A., Henning, G., Hofmann, D.K., and Benayahu, Y. Metamorphosis of *Heteroxenia fuscescens* planulae (cnidaria: octocorallia) is inhibited by crude oil: a novel short term toxicity bioassay. *Marine Environmental Research* 43 (4): 295-302, 1997.
- Lacher, T.E. and Goldstein, M.I. Tropical Ecotoxicology: status and needs. *Environmental Toxicology and Chemistry*. 16 (1): 100-111, 1997.
- Landrum, P.F. Bioavailability and toxicokinetics of polycyclic hydrocarbons sorbed to sediments for the amphipod *Pontoporeia hoyi*. *Environ. Sci. and Tech.* 23: 588-595, 1989.
- Lane, P. and Gross, S.S. Cell signalling by nitric oxide. *Seminars in Nephrology* 19 (3): 215-229, 1999.
- Leake, L. D. and Moroz, L. L. Putative nitric oxide synthase (NOS) containing cells in the central nervous system of the leech *Hirudo medicinalis*. *Brain. Res.* 723: 115-124, 1996
- Leake, L.D. and Moroz, L.L. Putative nitric oxide synthase (NOS)-containing cells in the central nervous system of the leech, *Hirudo medicinalis*: NADPH-diaphorase histochemistry. *Brain Research*. 723: 115-124, 1996.
- Lee, R.F. Metabolism of tributyltin by marine animals and possible linkages to effects. *Mar. Environ. Res.* 32: 29-35, 1991.
- Lesser, M.P. and Shick, J.M. Effects of irradiance and ultraviolet radiation on photoadaptation in the zooxanthellae of *Aiptasia pallida*: primary production, photoinhibition, and enzymatic defences against oxygen toxicity. *Marine Biology*. 102: 243-255, 1989.
- Lesser, M.P. Effect of ultraviolet radiation in algal-cnidarian symbioses: biochemical response and productivity of zooxanthellae. *American Zoologist*. 105A, 1998.
- Lesser, M.P. Effects of visible and ultraviolet radiation on the ultrastructure of zooxanthellae (*Symbiodinium*) in culture and in situ. *Cell Tissue Res.* 261: 501-508, 1990.

References

Lesser, M.P. Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnology and Oceanography*. 41 (2): 271-283, 1996.

Lesser, M.P. Oxidative stress causes coral bleaching during exposure to elevated temperatures. *Coral Reefs*. 16: 187-192, 1997.

Lesser, M.P. Photobiology of Natural populations of zooxanthellae from the sea anemone *Aiptasia pallida*: Assessment of the host's role in protection against ultraviolet radiation. *Cytometry*. 10: 653-658, 1989.

Lesser, M.P., Stochaj, W.R., Tapley, D.W., and Shick, J.M. Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs*. 8: 225-232, 1990.

Liew, F.Y. and Cox, F.E.G. Non-specific defence mechanism: the role of nitric oxide. *Immunoparasitology Today*. A17-A21, 1991.

Long, E.R. Ranges in chemical concentrations in sediments associated with adverse biological effects. *Marine Pollution Bulletin*. 24 (1): 38-45, 1992.

Longhurst, A.R. and Pauly, D. *Ecology of Tropical Oceans*. Academic Press, San Diego. 1987.

Lowe, D.M. and Pipe, R.K. Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. *Aquatic Toxicology*. 30: 357-365, 1994.

Lowe, D.M., Fossato, V.U., and Depledge, M.H. Contaminant- induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from Venice Lagoon: an *in vitro* study. *Marine Ecology Progress Series*. 129: 189-196, 1995a.

Lowe, D.M., Moore, M.N., and Evans, B.M. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limander limander*. *Marine Ecology Progress Series*. 91: 135-140, 1992.

References

- Lowe, D.M., Soverchia, C., and Moore, M.N. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquatic Toxicology*. 33, 105-112, 1995b.
- Madany, I.M., Al-Haddad, A., Jaffar, A. and Al-Shirbini. Spatial and temporal distributions of aromatic hydrocarbons in the coastal waters of Bahrain. *Arch. Environ. Contam. Toxicol.* 26: 185-190, 1994.
- Mance, G. Pollution Threat of Heavy Metals in Aquatic Environments, p. 372. Elsevier, Amsterdam. 1987.
- Martinez, A. Nitric oxide synthase in invertebrates. *Histochemical Journal*. 27: 770-776, 1995.
- Mascarelli, P.E. and Bunkley-Williams, L. An Experimental field evaluation of healing in damaged, unbleached and artificially bleached star coral, *Montastrea annularis*. *Bulletin of Marine Science*. 65 (2): 577-586, 1999.
- Matsumoto, T., Nakane, M., and Pollock, J. S. A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative *Neurosci. Lett.* 155:61-64, 1993.
- Matsumoto, T., Nakane, M., Pollock, J.S., Kuk, J.E., and Forstermann, U. A correlation between soluble brain nitric oxide synthase and NADPH-diaphorase activity is only seen after exposure of the tissue to fixative. *Neuroscience Letters*. 155: 61-64, 1993.
- McCloskey, L.R., Cove, T.G., and Verde, E.A. Symbiont expulsion from the anemone *Anthopleura elegantissima*. *J. Exp. Mar. Biol. and Ecol.* 195:173-186, 1996.
- McGuinness, K.A. Effects of oil spills on macro-invertebrates of salt marshes and mangrove forests in Botany Bay, NSW, Australia. *J. Exp. Mar. Biol. Ecol.* 142: 121-135, 1990.

References

- McKay, M. C. and Anderson, P. A. V. Preparation and properties of cnidocytes from the sea anemone *Anthopleura elegantissima*. *Biol. Bull.* 174: 47-53, 1988.
- McKinstry, M.J., Chapman, G.B., Spoon, D.M., and Peters, E.C. The occurrence of bacterial colonies in the epidermis of the tentacles of the sea anemone *Aiptasia pallida* (Anthozoa: Actinaria). *Tran. Am. Microscope. Soc.* 108 (3): 239-244, 1989.
- Meehan, W.J. and Ostrander, G.K. Coral bleaching: a potential biomarker of environmental stress. *J. of Tox. and Env. Health.* 50: 529-552, 1997.
- Mercier, A., Pelletier, E., and Hamel, J. F. Responses of temperate sea anemones to butyltin contamination. *Can. Jou. Fish. Aquat. Sci.* 55: 239-245, 1998.
- Mercier, A., Pelletier, E., and Hamel, J.F. Effects of butyltins on the symbiotic sea anemone *Aiptasia pallida* (Verill). *J. Exp. Mar. Biol. Ecol.* 215: 289-304, 1997.
- Mercier, A., Pelletier, E., and Hamel, J.F. Toxicological response of the symbiotic sea anemone *Aiptasia pallida* to butyltin contamination. *Marine Ecology Progress Series.* 144: 133-146, 1996.
- Miller, D., Brown, B., Sharp, V.A., and Nganro, N. Changes in the expression of soluble proteins extracted from the symbiotic anemone *Anemonia viridis* accompany bleaching induced by hyperthermia and metal stressors. *J. Therm. Biol.* 17 (4/5): 217-223, 1992.
- Moncada, S. and Higgs, A. The L-arginine-nitric oxide pathway. *The New England Journal of Medicine*: 2002-2012, 1993.
- Moncada, S., Palmer, R.M.J., and Higgs, E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews.* 43 (2): 109-142, 1991.
- Moore, H.B. Aspects of stress in the tropical marine environment. *Adv. Mar. Biol.* 10: 217-269, 1972.

References

- Moore, M.N. Cellular Responses to Pollutants. *Marine Pollution Bulletin*. 16 (4): 134-139, 1985.
- Moore, M.N. Lysosomal cytochemistry in marine environmental monitoring. *Histochem. J.* 22: 189-191, 1990
- Morgan, M.B. and Snell, T.W. Assessing coral stress at the level of gene expression. *American Zoologist*. 112A, 2000.
- Morgan, M.B., Vogelien, D.L. and Snell, T.W. Assessing coral stress responses using molecular biomarkers of gene transcription. *Environ. Toxicol. Chem.* 20 (3): 537-543, 2001.
- Moroz, L.L. and Gillette, R. From polyplacophora to cephalopoda: comparative analysis of nitric oxide signalling in mollusca. *Acta Biologica Hungarica*. 46 (2): 169-182, 1995.
- Morrall, C.E., Galloway, T.S., Trapido-Rosenthal, H.G. and Depledge, M.H. Characterisation of nitric oxide synthase activity in the tropical sea anemone *Aiptasia pallida*. *Comp. Biochem. and Physiol. B*. 125: 483-491, 2000.
- Morrall, C.E., Trapido-Rosenthal, H.G., Knap, A.H. and Depledge, M.H. Nitric oxide synthase as ecotoxicological biomarkers in the tropical marine environment. *Mar. Env. Res.* 46 (1-5): 429-432, 1998.
- Muller-Parker, G. Effect of Feeding regimen and irradiance on the photophysiology of the symbiotic sea anemone *Aiptasia pulchella*. *Marine Biology*. 90: 65-74, 1985.
- Muller-Parker, G. Photosynthesis-irradiance responses and photosynthetic periodicity in the sea anemone *Aiptasia pulchella* and its zooxanthellae. *Marine Biology*. 82: 225-232, 1984.
- Muller-Parker, G. Seasonal variation in light-shade adaption of natural populations of the symbiotic sea anemone *Aiptasia pulchella* (Carlgreen, 1943) in Hawaii. *J. Exp. Mar. Biol. Ecol.* 112: 165-183, 1987.

References

- Muller-Parker, G., Cook, C.B., and D'Elia, C.F.D. Feeding affects phosphate fluxes in the symbiotic sea anemone *Aiptasia pallida*. *Marine Ecology Progress Series*. 60: 283-290, 1990.
- Munzel, T. Heitzer, T. and Harrison, D. G. The physiology and pathophysiology of nitric oxide superoxide system. *Herz*. 22: 158-172, 1997.
- Muscatine, L., Grossman, D., and Doi, J. Release of symbiotic algae by tropical sea anemones and corals after cold shock. *Marine Ecology Progress Series*. 77: 233-243, 1991.
- Nathan, C. and Hibbs, J.B. Role of nitric oxide in macrophage antimicrobial activity. *Current Opinion in Immunology* 3: 65-70, 1991.
- Nathan, C. and Xie, Q. Nitric Oxide Synthases: Roles, Tolls and Controls. *Cell*. 78: 915-918, 1994.
- Nathan, C. and Xie, Q. Regulation of biosynthesis of nitric oxide. *Journal of Biological Chemistry* 269: 13725-13728, 1994.
- National Research Council (NRC) Committee on biological markers. *Environ. Health. Perspect.* 74, 3-9, 1987.
- Needler, M. and Ross, D.M. Neuromuscular activity in sea anemone *Calliactis parasitica* (Couch). *JMBA.UK*. 37: 789-805, 1958.
- Nii, C.M. and Muscatine, L. Oxidative stress in the symbiotic sea anemone *Aiptasia pulchella* (Carlgreen, 1943): Contribution of the animal to superoxide ion production at elevated temperature. *Biol. Bull.* 192: 444-456, 1997.
- Omori, M., Fukami, H., Kobinata, H. and Hatta, M. Significant drop of fertilization of *Acropora* corals in 1999: An after-effect of heavy coral bleaching? *Limnol. Oceanogr.* 46 (3): 704-706, 2001.

References

- Ottaviani, E. and Franceschi, C. A new theory on the common evolutionary origin of natural immunity, inflammation and stress response: the invertebrate phagocytic immunocyte as an eyewitness. *Domestic Animal Endocrinology*. 15 (5): 291-269, 1998.
- Ottaviani, E. and Franceschi, C. The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems. *Immunol. Today*. 18: 169-174, 1997.
- Ottaviani, E., Franchini, A., Cassanelli, S., and Genedani, S. Cytokines and invertebrate immune responses. *Biol. Cell*. 85: 87-91, 1995.
- Ottaviani, E., Paemen, L.R., Cadet, P., and Stefano, G.B. Evidence for nitric oxide production and utilisation as a bactericidal agent by invertebrate immunocytes. *European Journal of Pharmacology (Environmental Toxicology and Pharmacology Section)* 248: 319-324, 1993.
- Palincsar, E.E., Jones, W.R., Palincsar, J.S., Glogowski, M. A. and Mastro, J. L. Bacterial aggregates within the epidermis of the sea anemones *Aiptasia pallida*. *Biol. Bull.* 177, 130-140, 1989.
- Palmer, R.M.J., Ashton, D.S., and Moncada, S. Vascular endothelial cells synthesise nitric oxide from L-arginine. *Nature*. 333: 664-666, 1988.
- Peachey, R.L. and Crosby, D.G. Phototoxicity in tropical reef animals. *Marine Environmental Research*. 42 (1-4): 359-362, 1996.
- Pearse, V. Modification of sea anemone behaviour by symbiotic zooxanthellae: expansion and contraction. *Biol. Bull.* 147: 641-651, 1974.
- Peters, E.C. A survey of cellular reactions to environmental stress and disease in Caribbean scleractinian corals. *Hegol. Meeresunters.* 37: 113-137, 1984.

References

- Peters, E.C., Gassman, N.J., Firman, J.C., Richmond, R.H., and Power, E.A. Ecotoxicology of tropical marine ecosystems. *Environmental Toxicology and Chemistry* 16 (1): 12-40, 1997.
- Peters, E.C., Oprandy, J.K. and Yevich, P.P. Possible causal agent of 'White Band Disease' in Caribbean acroporid corals. *J. Invertebrate. Pathol.* 41: 394-396, 1983.
- Peterson, D.A., Peterson, D.C., Archer, S., and Weir, E.K. The non-specificity of specific nitric oxide synthase inhibitors. *Biochemical and Biophysical Research Communications* 187 (2): 797-801, 1992.
- Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H., Nakane, M. and Murad, F. Purification and characterisation of particulate endothelium derived relaxation factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA.* 88:10480-10484, 1991.
- Powell, E.N., Kendall, J.J., Connor, S.J., Zastrow, C.E., and Bright, T.J. Effect of eight outer continental shelf drilling muds on the calcification rate and free amino acid pool of the coral *Acropora cervicornis*. *Bull. Environ. Contam. Toxicol.* 33: 362-372, 1984.
- Prezelin, B.B. Light reactions in photosynthesis. Physiological bases of phytoplankton ecology. *Can. Bull. Fish. Aquat. Sci.* 210: 1-43, 1981.
- Prince, R.C. and Gunson, D.E. Rising interest in nitric oxide synthase. *TIBS.* 18: 35-36, 1993.
- Prior, R.L. and Cao, G. *In vivo* total antioxidant capacity. Comparison of different analytical methods. *Free Radical Biology and Medicine.* 27: 1173-1181, 1999.
- Radomski, M.W., Palmer, R.M.J., Moncada, S. Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br. J. Pharmacol.* 101: 325-328, 1990.

References

- Radomski, W., Martin, J. F., and Moncada S. Synthesis of nitric oxide by the haemocytes of the American horseshoe crab (*Limulus polyphemus*). *Phil. Trans. R. Soc. Lond. B.* 334: 129-133, 1991.
- Regoli, F., Nigro, M., and Orlando, E. Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology*. 40: 375-392, 1998a.
- Regoli, F. and Winston, G.W. Applications of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. *Marine Environmental Research* 46 (1-5): 439-442, 1998b.
- Reichelt-Brushett, A.J. and Harrison, P.L. The Effect of copper, Zinc and cadmium on fertilisation success of gametes from scleractinian reef corals. *Marine Pollution Bulletin*. 38, 182-187, 1999.
- Rice-Evans, C. and Halliwell, B. *Preface*. In: Free radicals and oxidative stress; environmental, drugs, and food additives, edited by Rice-Evans, C., Halliwell, B., and Lunt, G.G. Portland Press. vii-viii, 1994.
- Ringwood, A.H., Connors, D.E., and Hoguet, J. Effects of natural and anthropogenic stressors on lysosomal destabilisation in oysters *Crassostrea virginica*. *Marine Ecology Progress Series*. 166: 163-171, 1998.
- Roberts, C.M., McClean, C.J., Veron, J.E.N., Hawkins, J.P., Allen, G.R., McAllister, D.E., Mittermeier, C.G., Schueler, F.W., Spalding, M., Wells, F., Vaynne, C. and Werner, T.B. Marine biodiversity hotspots and conservation priorities for tropical reefs. *Science*. 295: 1280-1284, 2002.
- Roughley, T.C. In: Wonders of the Great Barrier Reef. 13th edition. Angus and Robertson, Sydney. 1954.
- Rowen, R. and Knowlton, N. Intraspecific diversity and ecological zonation in coral-algal symbiosis. *Proc. Natl. Acad. Sci. USA*. 92: 2850-2853, 1995.

References

- Sadiq, M. Toxic Metal Chemistry in Marine Environments, p 390. Marcel Dekker, New York. 1992.
- Salleo, A., Musci, G., Barra, P.F.A., and Calabrese, L. The discharge of acontial nematocytes involves the release of nitric oxide. *Journal of Experimental Biology*. 199: 1261-1267, 1996.
- Saunders, B.K. and Muller-Parker, G. The effects of temperature and light on two algal populations in temperate sea anemone *Anthopleura elegantissima*. *Jou. Exp. Mar. Biol. and Ecol.* 211: 213-224, 1997.
- Schmidt, H. H. H. W., Hofmann, H., Schindler, U., Shutenko, Z.S., Cunningham, D.D. and Feelisch, M. No NO from NO synthase. *Proc. Natl. Acad. Sci. USA*. 93: 14492-14497, 1996.
- Schmidt, H.H. and Walter, U. NO at work. *Cell*. 78: 919-925, 1994.
- Schmidt, H.H.H.W. and Kelm, M. *Determination of nitrite and nitrate by the Greiss reaction*. In: *Methods in NO Research*. Eds. Feelisch, M. and Stamler, J.S. Wiley. 491-497, 1997
- Schmidt, K., List, B.M., Klatt, P., and Mayer, B. Characterisation of Neuronal amino acid transporters: uptake of nitric oxide synthase inhibitors and implications for their biological effects. *Journal of Neurochemistry*. 64 (4): 1469-1475, 1995.
- Searle, J., Kerr, J.F.R. and Bishop, C.J. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol. Annu.* 17: 229-259, 1982.
- Seligman, P.F., Valkirs, A.O. Stan, P.M. and Lee, R.F. Evidence of rapid degradation of tributyltin in a marina. *Mar. Poll. Bull.* 19: 531-534, 1988.
- Sharp, V.A., Miller, D., Bythell, J.C., and Brown, B.E. Expression of low molecular weight HSP 70 related polypeptides from the symbiotic sea anemone *Anemonia viridis* Forskall in response to heat shock. *J. Exp. Mar. Biol. Ecol.* 179: 179-193, 1994.

References

- Sheehan, D. and Power, A. Effects of seasonality on xenobiotic and antioxidant defence mechanisms of bivalve molluscs. *Comp. Biochem. and Phys.* 123C: 193-199; 1999.
- Shelton, G. A. B. *Electrical Conduction and Behaviour in Simple Invertebrates*. Oxford University Press. New York. 203-242, 1982.
- Shick, J. M. *A Functional Biology of Sea Anemones*. Edited by Calow, P. Chapman and Hall. 1-395, 1991.
- Shick, J.M. and Dykens, J.A. Photobiology of the symbiotic sea anemone, *Anthopleura elegantissima*: photosynthesis, respiration, and behaviour under intertidal conditions. *Biol. Bull.* 166: 608-619, 1984.
- Shick, J.M., Lesser, M.P., Dunlap, W.C., Stochaj, W.R., Chalker, B.E., and Won, J.W. Depth-dependent responses to solar ultraviolet radiation and oxidative stress in the zooxanthellate coral *Acropora microphthalmus*. *Marine Biology* 122: 41-51, 1995.
- Slater, T.F. *Biochemical studies on liver injury*. In: Biochemical Mechanisms of Liver Injury. Ed. Slater, T.F. Academic Press, London, New York. 1-44, 1978.
- Smith, G.J., Weis, V., and Muscatine, L. The cell cycle of symbiotic dinoflagellates: the effects of darkness and photosynthetic inhibitors in *in situ* cell division by zooxanthellae in the sea anemone *Aiptasia pallida*. *Journal of Phycology* 24: 17, 1988.
- Smith, H.G. The significance of the relationship between actinians and zooxanthellae. *J. Exp. Biol.* 16, 334-345, 1939.
- Smith, K. L. Galloway, T. S. and Depledge, M. H. Neuro-endocrine biomarkers of pollution-induced stress in marine invertebrates. *Sci. Total. Environ.* 262: 185-190, 2000.
- Smith, V. J. and Hutton, D. M. *Measurement of extracellular reactive oxygen species in Anthozoans and other marine invertebrates by cytochrome C reduction*. In, Techniques in Fish Immunology (IV). Eds. Stolen, J. S., Fletcher, T. C., Smith, S. A., Zelikoff, J. T.,

References

- Kaattari, S. L., Anderson, R. S., Soderhall, K. and Weeks, B. A. SOS Publications, Fair Haven N J, USA. 27-35. 1995.
- Smith, V.J. and Hutton, D.M.C. Measurement of extracellular reactive oxygen species in anthozoans and other marine invertebrates by cytochrome C reduction. *Techniques in Fish Immunology*. 4: 27-35, 1995.
- Snyder, S.H. More jobs for that molecule. *Nature*. 372: 504-505, 1994.
- Snyder, S.H. and Brecht, D.S. Biological roles of Nitric Oxide. *Scientific American*. 260 (5): 28-35, 1992.
- Sono, M., Roach, M. P., Coulter, E. D. and Dawson, J. H. *Chem. Rev.* 96: 2841-2848, 1996.
- Stamler, J.S. Redox Signalling: nitrosylation and related target interactions of nitric oxide. *Cell*. 78: 931-936, 1994.
- Stebbing, A.R.D. and Brown, B.E. *Marine ecotoxicological tests with coelenterates*. In: Ecotoxicological testing for the marine environment. Eds. Persoone, G., Jaspers, E., and Claus, C. 307-339, 1984.
- Stebbing, A.R.D. Organotins and water quality-some lessons to be learned. *Mar. Poll. Bull.* 16 (10): 383-390, 1985.
- Steen, R.G. and Muscatine, L. Low temperature evokes rapid exocytosis of symbiotic algae by a sea anemone. *Biology Bulletin*. 172: 246-263, 1987.
- Stegeman, J.J., Brouwer, M., Di Giulio, R.T., Forlin, L., Fowler, B.A., Sanders, B.M., and Van Veld, P.A. *Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect*. In: Biomarkers Biochemical. Physiological and histological markers of anthropogenic stress. Eds. Huggett, R.J., Kimerle, R.A., Mehrle, P.M., and Bergman, H.L. SETAC. 235-335, 1992.

References

- Sterrerr, W. How Many Species are there in Bermuda? *Bulletin of Marine Science*. 62 (3): 809-840, 1998.
- Sterrerr, W. Marine Flora and Fauna of Bermuda. Wiley-Interscience. 1986.
- Stewart, C. and Thompson, J.A.J. Extensive butyltin contamination in south-western coastal British Columbia. *Mar. Poll. Bull.* 28 (10): 601-606, 1994.
- Stochaj, W.R. and Grossman, A.R. Differences in the protein profiles of cultured and endosymbiotic symbiodinium sp. from the anemone *Aiptasia pallida*. *J. Phycol.* 33: 44-53, 1997.
- Strasser, R.J. *A concept for stress and its application in remote sensing*. In: Applications of chlorophyll fluorescence. Ed. Lichtenthaler. Kluwer Academic Publishers, The Netherlands. 333-337, 1988.
- Stuehr, D.J. and Griffith, O.W. Mammalian nitric oxide synthases. *Advances in Enzymology*. 65: 287-347, 1992.
- Stuehr, D.J. and Marletta, M.A. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon. *The Journal of Immunology* 139 (2): 518-525, 1987.
- Sutton, D.C. and Hoegh-Guldberg, O. Host-zooxanthallae interactions in four temperate marine invertebrate symbioses: Assessment of effect of host extracts on symbionts. *Biol. Bull.* 178: 175-186, 1990.
- Tapley, D.W. Photoinactivation of catalase but not superoxide dismutase in the symbiotic sea anemone *Aiptasia pallida*. *American Zoologist*. 53A, 1998.
- Tapley, D.W., Shick, J.M., and Smith, J.P.S. Defences against oxidative stress in the symbiotic sea anemone *Aiptasia pallida* and *Aiptasia pulchella*. *American Zoologist*. 105A, 575, 1998.

References

- Thomas, M.L.H., Eakins, K.E., and Logan, A. Physical characteristics of the anchialine ponds of Bermuda. *Bulletin of Marine Science*. 48 (1): 125-136, 1991.
- Thompson, Jr. J. H., Shinn, E. A. and Bright, T. J. In: *Marine Environmental Pollution. I. Hydrocarbons*. Ed. Geyer, R.A. Elsevier Oceanography series, 27A, Elsevier, Amsterdam, 433-453, 1980.
- Tracey, W.R. Spectrophotometric detection of nitrogen oxides using azo dyes. *Neuroprotocols: A Companion to Methods in Neurosciences*. 1 (2): 125-131, 1992.
- Trapido-Rosenthal, H.G., Holder, H.A., Fraser-Smith, L.R., and Billinghamurst, Z. Possible involvement of arginine and nitric oxide in the chemical mediation of symbiotic relationships between photosynthetic dinoflagellate algae and anthozoans. *Chemical Senses* 21 (XV111 abstracts): 681-682, 1996.
- Trapido-Rosenthal, H.G. Sharp, K.H. Morrall C.E. and Galloway, T.S. Nitric oxide and cnidarian-dinoflagellate symbioses: pieces of a puzzle. *Amer. Zool.* 41: 247-257, 2001.
- Trench, R.K. Microalgal-Invertebrate symbiosis: A Review. *Endocytobiosis and Cell Res.* 9: 135-175, 1993.
- Trottier, S., Kusui, T., Blaise, C., and Johnson, E.M. Acute toxicity assessment of aqueous samples using a microplate based *Hydra attenuata* assay: technical methodology. *Environmental Toxicology and water quality* 12 (3), 1997.
- Tsimilli-Michael, M., Pecheux, M., and Strasser, R.J. Light and heat stress adaption of the symbionts of temperate and coral reef foraminifers probed in hospite by the chlorophyll a fluorescence kinetics. *Journal of Biosciences*. 54 (9-10): 671-680, 1999.
- Twain, M. From a letter to Elizabeth Wallace. 1910.
- Verill, V. E. Additions to the Anthozoa and Hydrozoa of the Bermudas. *Trans. Conn. Acad. Arts. Sci.* 10: 551-572, 1900.

References

- Verill, V. E. *The Bermuda Islands*. Part V. Characteristic life of the Bermuda coral reefs. *Trans. Conn. Acad. Arts. Sci.* 12: 204-348, 1907.
- Wang, J.T. and Douglas, A.E. Essential amino acid synthesis and nitrogen recycling in an alga-invertebrate symbiosis. *Marine Biology*. 135: 219-222, 1999.
- Warner, M.E. Fitt, W.K. and Schmidt, G.W. The effects of elevated temperature on the photosynthetic efficiency of zooxanthellae *in hospite* from four different species of reef coral: a novel approach. *Plant Cell and Environ.* 19: 291-299, 1996.
- Warner, M.E., Fitt, W.K., and Schmidt, W. Damage to photosystem II in symbiotic dinoflagellates: a determinant of bleaching. *Proc. Natl. Acad. Sci.* 96: 8007-8012, 1999.
- Weinberg, R.J., Valtschanof, J.G., and Schmidt, H.H.H.W. *The NADPH Diaphorase Histochemical stain*. In: *Methods in NO Research*. Eds. Feelisch, M. and Stamler, J.S. Wiley. 237-247. 1997.
- Weis, V. and Levine, R.P. Differential protein profiles reflect the different lifestyles of symbiotic and aposymbiotic *Anthopleura elegantissima*, a sea anemone from temperate waters. *The Journal of Experimental Biology*. 199: 883-892, 1996.
- Wesseling, I., Uychiaoco, A.J., Alino, P.M., Aurin, T., and Vermaat, J.E. Damage and recovery of four Philippine corals from short-term sediment burial. *Mar. Ecol. Prog. Ser.* 176: 11-15, 1999.
- Wilkerson, F.P. and Muscatine, L. Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. *Proc. R. Soc. Lond.* 221:71-86, 1984.
- Wink, D.A., Vodovotz, Y., Grisham, M.B., DeGraff, W., Cook, J.C., Pacelli, R., Krishna, M., and Mitchell, J.B. Antioxidant effects of nitric oxide. *Methods In Enzymology* 301: 413-425, 1999.

References

- Winston, G. W., Moore, M. N., Straatsburg, I. And Kirchin, M. A. Decreased stability of digestive gland lysosomes from the common mussel *Mytilus edulis* by *in vitro* generation of oxygen-free radicals. *Arch. Environ. Toxicol.* 21: 401-408, 1991.
- Winston, G.W. and Di Giulio, R.T. Peroxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*. 19: 137-161, 1991.
- Winston, G.W., Mayeaux, M.H., and Heffernan, M. Benzo [a] pyrene metabolism by the intertidal sea anemone, *Bunodosoma caverata*. *Marine environmental research*. 45 (1): 89-100, 1998.
- Xia, Y. and Zweier, J. L. Direct measurement of nitric oxide synthase. *Proc. Natl. Acad. Sci. USA*. 194, 12705-12710, 1997.
- Xia, Y., Dawson, V.L., Dawson, T.M., Snyder, S.H., and Zweier, J.L. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. USA*. 93: 6770-6774, 1996.
- Zamer, W.E., Shick, J.M., and Tapley, D.W. Protein measurement and energetic considerations: comparisons of biochemical and stoichiometric methods using bovine serum albumin and protein isolated from sea anemones. *Limnol. Oceanogr.* 34 (1): 256-263, 1989.
- Zhao, C., Liaw, L., Lee, I.H., and Lehrer, R.I. cDNA cloning of clavanins: antimicrobial peptides of tunicate haemocytes. *FEBS*. 410: 490-492, 1997.